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STUDIES ON THE BLOCK STRUCTURE OF THE CORNEAL EPITHELIUM, WITH REFERENCE TO THE MECHANISM FOR DEVELOPMENT OF THE DENDRITIC KERATITIS

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The superficial herpes simplex keratitis shows usually a dendritic figure on the cornea, while the same virus does not make such a peculiar lesion on other tissues of the body. It must be due to the characteristic structure of the epithelial layer of the cornea. The present study was undertaken in an attempt to clarify this problem by the observation of the experimental dendritic keratitis of rabbits.

Materials and Methods

Both full-grown and child rabbits were used. After one or two linear superficial incisions of about 3 to 5 mm length were made on the cornea, the inoculums were instillated onto the wound. When the dendritic lesion developed, the affected cornea was removed and fixd in 10% neutral formalin solution. Some of the fixed materials were observed by means of microscope as either a whole layer or a superficial partial layer preparation, and the others were observed in section after hematoxylin-eosin stain, PAS stain, azan stain, and silver impregnation. Some materials were observed after methylenblue or hematoxylin vital staining.

The inoculum of the herpes simplex virus was FL-HL 3 strain, SUGIYAMA strain and KOSEKI strain. Both the purified vaccinia virus and the croton oil were used as controls of the inoculum in this study.

Results

In many cases of the rabbits which have undergone two parallel linear incisions, the characteristic keratitis was caused from 3 to 7 days after the inoculation.

The elementary types of the superficial herpes simplex keratitis.

At first, several knob-like opacities appeared on the linear wound of the inoculation. These knob-like opacities were at almost the same distance, one by one on the linear wound, wherever the wound was located. (Fig. 1) In a few cases a knob-like opacity disappeared without any successor. (Fig. 2) But in most cases, a new linear lesion developed from the first knob just as a branch growing from the linear wound-trunk, and then, the second knob appeared at the free end of that branch line. Thus, the first and the second knobs were connected with the new linear lesion, making a dumb-bell like figure. (Fig. 3) In a short time, the second linear lesion grew to a different direction from the second knob, and the third knob appeared at the end of this new

branch. Thus the lesion grew more and more and finally a dendritic figure was made. (Fig. 4)

It is evident, that the elementary types of the superficial herpes simplex keratitis are both a knob-like and a linear lesion, and that the dendritic figure was produced by the combination of these two types of lesion.

Microscopic observations of the superficial herpes simplex keratitis.

The characteristic cellular alterations induced by the herpes simplex virus were found in the epithelial cells not only at the knob-like lesion but also at the linear one. (Fig. 8) The most outstanding finding in the flat specimen was several fissures running radially out from the center of the knob. In preference they might be called "channel". A single knob showed often a star like figure, because of this structure. (Figs. 5 and 6) In the axis of the linear lesion, there was a similar single channel which contained often many wandering cells marching in a file. (Fig. 7)

The ceannels, if they were called so, had not any specific walls and were formed by the communication of enlarged intercellular spaces.

Several radiating channels were observed in the knob-like lesion. while only one channel was found in the axis of the linear lesion. These observations suggested, that the places of the knob corresponded to the conflux of the channels.

Distance between the two neighbouring knob-like lesions.

The distance between the two neighbouring knob-like lesions which developed on the linear wound was measured 0.20 to 0.52 mm, 0.36 mm on an average, while that of the two neighbouring knobs of the dendritic figure was measured 0.18 to 0.63 mm, and the mean was 0.35 mm. (Fig. 13) There was a good agreement in value between the two kinds of lesion.

Observation of the vaccinia keratitis.

If such a channel existed in the corneal epithelium, it might be seen not only in the herpetic keratitis, but also in other inflammations of the corneal epithelium. Then, the experimental vaccinia keratitis and the keratitis due to the application of croton oil were observed.

The vaccinia keratitis had a more diffuse character, than the herpetic one, but it showed occasionally linear figures in the early stage, as shown in Fig. 11. The width of the spaces marginated with the vine-like lesions in Fig. 11 was measured 0.22 to 0.77 mm, on an average 0.40 mm. This value agreed nearly with that of the internodular distances in the herpetic keratitis. There was also a similar single channel in the axis of the linear lesion of the vaccinia keratitis.

Observation of the keratitis due to the croton oil application.

The croton oil was instillated onto the linear wound of the cornea and forty eight hours later the hematoxylin saline solution was injected into the corneal parenchyma. Then, the pigment invaded into the basal cell layer of the epithelium, as shown in Fig. 10, making a net work.

A deeply stained and relatively wide line of the network might be a channel situated in the basal cell layer. (Fig. 10a) One of such lines had contact with the nerve canal which contained the same pigment and ran through the parencyma to the Bowman's membrane.

The fluid running from the parenchyma might pour into the channel of the epithelium at the hole of the Bowman's membrane.

A similar result was obtained by the injection of croton oil into the corneal parenchyma.

Discussion

The skin and the mucous membaane, such as the mouth's lip, oral cavity and genitals, can be affected with herpes simplex virus and vesicles are formed on those tissues, but the dendritic figure as shown on the cornea, is not caused by the same virus.

It is likely, that the development of the dendritic figure may have any relation with the structure of the corneal epithelium.

When herpes simplex virus is inoculated onto the linear wound of the superficial layer of the cornea, knob-like lesions develop at a similar distance on that line.

It is presumed, that there are areas which are especially easy to be affected with the herpes simplex virus, and that these areas are at a similar distance in the corneal epithelium.

It must be due to the structure of the tissue, since each epithelial cell at the same depth may have not any difference about the sensitivity for the same virus.

When the development of the dendritic figure is observed in detail, at first a knob-like lesion appears, then a line of opacity grows and it ends in the second knob. These knobs develop at a regular distance of about 0.35 mm with one another. The characteristic cellular changes are found not only at the knob but also at the line. Therefore, it is evident that both kinds of lesion are induced by the herpes virus infection.

The knob is considered to locate at the conflux of the channels, since the knob has several radiating channels in itself and is a bending point or a point of bifurcation of dendritic figures.

From these observations, it may be sure that the corneal epithelium consists of blocks which sre formed by colonies of cells, 0.3 to 0.4 mm in the diameter, and that the outside of the blocks is surrounded by a narrow channel.

The herpes simplex virus must prefer to affect the epithelial cells along these channels and the dendritic figure is produced in the result.

Such a structure of the epithelium is shown schematically in Text-Fig, 1. In this picture, XX′ is a incised wound of inoculation, A is a crossing of XX′ with channels, and B is a conflux of channels. The knobs which appear on the line XX′ may be

situated at the point A, and the knobs which develop in the dendritic keratitis may locate at the point B.

These channels are formed by the communication of enlarged intercellular spaces and has not any specific wall. It is evident that the channels are no artifact, because many wandering cells are frequently found to march in it in a file. It is presumed that these channels exist at a normal state and are not produced secondarily by the inflammation, since such channels are found not only in the herpes simplex keratitis but also in the vaccinia keratitis and in the simple keratitis due to the croton oil application, and moreover knob-like or linear lesions develop at the regular distance on the cornea.

In other words, such channels exist surely in the cornea, but are invisible in the normal state. When virus infection or any inflammation occurs, such channels become visible by containing wandering cells or any alterations of the surrounding cells.

The fluid which has entered into the epithelial layer may accumulate at first in those channels running around the cell blocks, then may invade each intercellular space.

These channels may shut in the rest stage and open in the active stage and thus may act as the main path way of fluid in the epithelium.

Those channels, therefore, may be adequately called "potential channel".

The networks of the potential channel are present on the parallel planes to the surface of the cornea, at the depth of the basal cell layer and slightly more shallow layer of the epithelium but it is not evident whether or not only two systems of the channel exist.

The herpetic lesion develops usually on the same plane, wheras the lesion caused by the vaccinia virus has a tendency to spread to the different planes at the same time. One of the latter case is shown in Fig. 12, in which the two patterns of the network of the channel are different with each other.

Such a structure is reasonable to keep the strength of the epithelium. It is not obvious in which way the networks of the channel at the different planes are connected.

The pattern of the network of the channel may be changed step by step, because the epithelial cells of the cornea are taken off from the outer surface by and by. Two different patterns as shown in Fig. 12 suggest it. However, the channel in the basal cell layer must be prescribed in some degree by the existence of the hole at the Bowman's membrane.

In other words, the conflux of the channel in the basal cell layer may correspond to the site of the hole at the Bowman's membrane through which the nerve and fluid enter into the epithelium from the parenchyma, but it wants to be confirmed.

The block structure of the corneal epithelium is shown schematically in Text-Fig. 2. In this picture the part of the hole at the Bowman's membrane is drawn according to that presumption.

Fig. 1

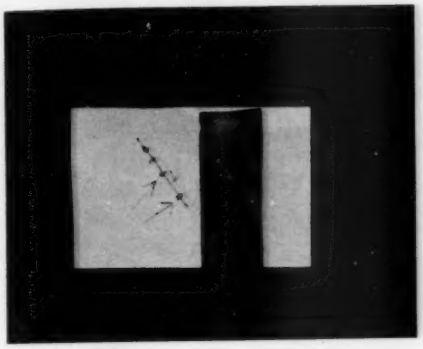


Fig. 2

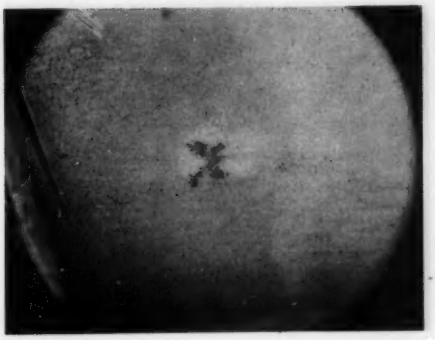


Fig. 3

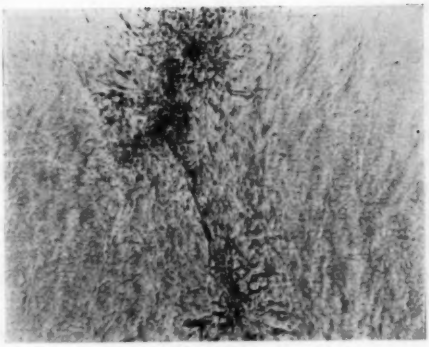
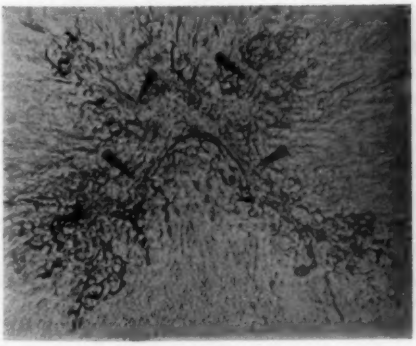


Fig. 4

Fig. 5



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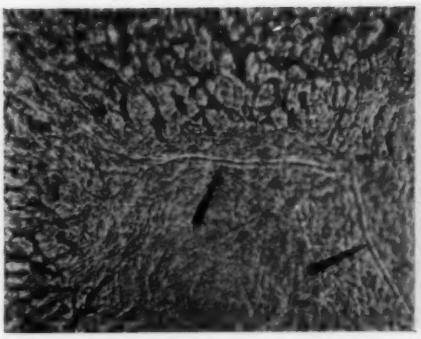


Fig. 7

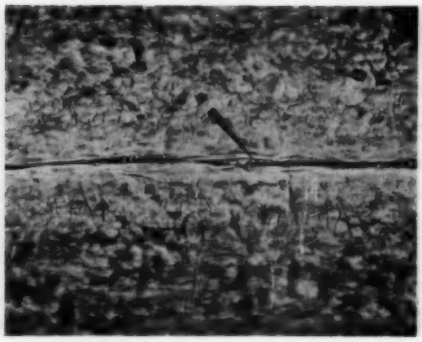


Fig. 8

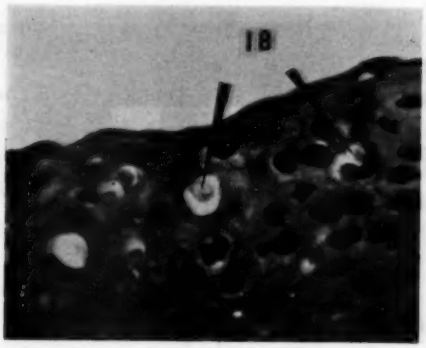


Fig. 9

Fig. 10

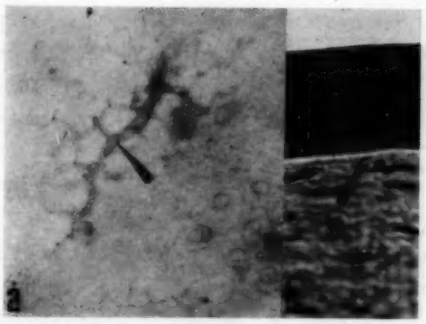


Fig. 11

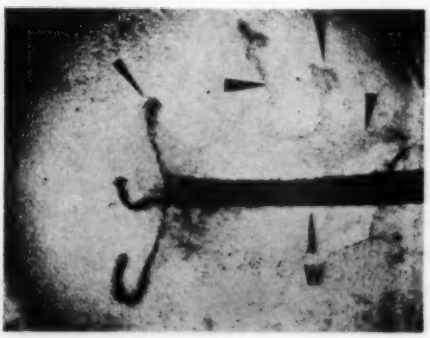
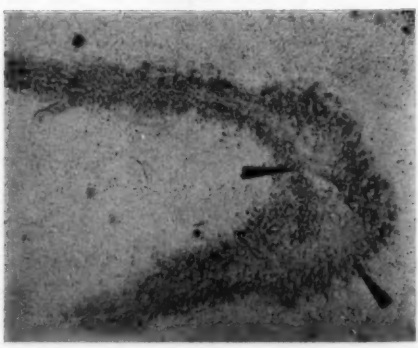


Fig. 12



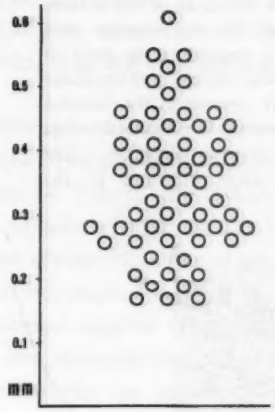


Fig. 13. Distribution of the distance between the two neighbouring knob-lke lesions in the rabbit's cornea infected with herpes simple virus.

Explanation of Figures

Fig. 1 Knob-like lesions which are arraanged in the similar distance on the linear wound of rabbit's cornea infected with herpes simplex virus. Biomicroscopic photograph.

Fig. 2 A single knob-like lesion which developed in the rabbit's cornea infected with herpes simplex virus. Flat specimen. No stain.

Fig. 3 A dumb-bell like lesion which developed in the rabbit's cornea infected with herpes simplex virus. Flat specimen. No stain.

Fig. 4 A dendritic lesion which developed in the rabbit's cornea infected with herpes simplex virus. Flat specimen. Methylenblue stain.

Fig. 5 Activated potential channels (Arrow) which run radially in the knob like lesion of the rabbit's cornea infected with herpes simplex virus. Flat specimen. No stain.

Fig. 6 Activated potential channels (Arrow) which run radially in the knob-like lesion of the rabbit's cornea infected with herpes simplex virus. Flat specimen. No stain.

Fig. 7 An activated potential channel which runs between the two neighbouring knob-like lesions, containing round infiltration cells (Arrow). Flat specimen. No stain.

Fig. 8 Eosinophilic inclusion bodies found in the epitelial cells which situated along the potential channel between the two neighbouring knob-like lesions in the rabbit's cornea infected with herpes simplex virus. H-E stain.

Fig. 9 Edemotous swelling of the subepithelial layers which situated beneath the linear epithelial lesion between the two neighbouring knob-like ones. H-E stain.

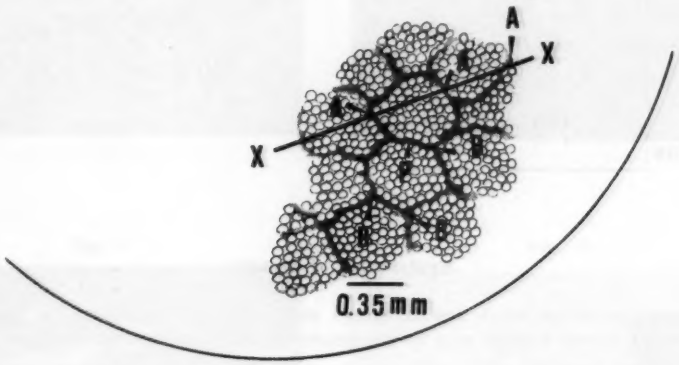
Fig. 10(a) An enlarged intercellular spaces (Arrow) which were jointed together to make a line in the epithelial basal cell layer of the rabbit's cornea, after instillation of croton oil. Two percent of hematoxylin solution was injected into the corneal stroma, 48 hours later. This line may correspond to an activated potential channel. Flat specimen.

(b) Bowman's membrane of the rabbit's cornea (Arrow). H-E stain.

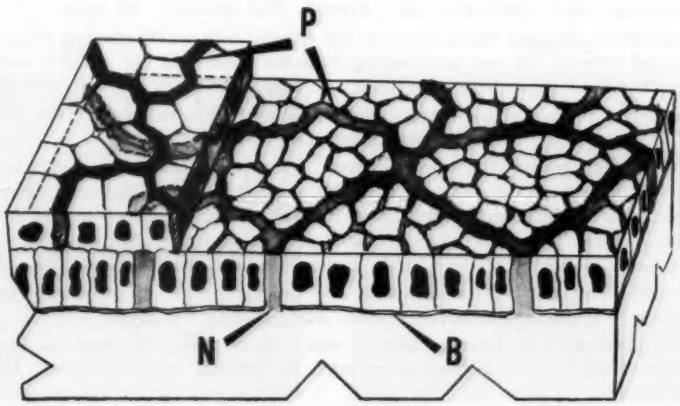
Fig. 11 Vine-like lesions which developed in the rabbit's cornea infected with vaccinia virus (Arrow). W:Linear inoculation wound. Flat specimen. No stain.

Fig. 12 Two systems of the potential channel which are placed on the deep and middle epithelial layers of the rabbit's cornea and activated by vaccinia virus infection. Flat specimen. No Stain.

Text-Fig. 1 Horizontal view of the block structure of the corneal epithelium. Potential channels (P) run between each block of the epithelial cell. A represents the point of the intersection of the potential channel and the linear inoculation wound (XX'). B represents the junction of the potential channels. The knob-like lesions develope at the point A or B in herpetic Keratitis. The diameter of each block is about 0.35 mm in the rabbit's cornea.



Text-Fig. 2 Stereographic representation of the block structure of the corneal epithelium. Two systems of the potential channel (Arrow) are placed on the parallel horizontal planes in the epithelial layers. The junction of the potential channels in the basal cell layer may corresponds to the entrance of the nerve at the Bowmans membrane. N: Entrance of the nerve. B: Bowman's membrane.



A similar structure is also found in the bile capillary of the liver. In that capillary the bile runs through the channel surrounded by the parenchymatous cells, and it is told, that the channel may shut in the rest stage and open in the active stage.

The differnce of the character between the herpes simplex keratitis and the vaccinia keratitis was briefly described above.

It must be due to the property of these two viruses. Both of the viruses cause the intra-and intercellular edema, but the herpes simplex virus produces more severe intercellular edema, than the other kind and this ability is the cause of the characteristic vesicle formation.

The herpes simplex virus may be taken off by the current of the exsudate which fills up in the potential channel of the epithelium in the early stage of lesion and may invade successively the epithelial cells along the channel, and as a result the dendritic figure may be produced.

The knob-like opacity of the dendritic figure may be the lesion at the conflux of the channels.

The vaccinia virus also affects the epithelial cells along the potential channel in the early stage and forms a linear opacity, but the lesion extends soon in all direction to form a diffuse keratitis.

The pathogenicity and the affinity for the tissue of the herpes simplex virus are said to have some differences between the strains. But the three strains examined in this study showed no difference in the figure of keratitis.

It is presumed that the direction of the current in the potential channel may be variable under the inflammatory condittion and may make itself at times inverse a shown in the blood vessels of the bulbar conjunctiva.

The characteristic structure as above described may be more adequately called as segmentary or a lobular structure as a medical term. But concerning the corneal epithelium, it appears to be alright to call it "block structure", because it is just like the block of a street in the horizontal view. However, when the word "block structure" is not suitable as a medical term, it will be replaced with an other word.

Summary

With rabbits, The superficial herpes simplex keratitis, the vaccinia keratitis and the keratitis due to the instillation and injection of croton oil were investigated. Consequently, it was surely proved that the corneal epithelium had a block structure.

The corneal epithelium consists of blocks which are formed by colonies of cells, 0.3-0.4 mm in diameter. The outside of the block is surrounded by a narrow channel. The chrnnel is formed by the communication of intercellular spaces. The channel may shut in the rest stadium and open in the active stadium. It is presumed that the channel acts as a passage of the fluid. The authors named it "Potential channel".

The potential channel is present on the parallel plane to the surface of the cornea, at the depth of the basal cell layer of the epithelium, and slightly more shallow layer.

The elementary types of the superficial herpes simplex keratitis are the knob-like lesion in the corneal epithelium and the linear lesion which connects the knobs. The dendritic keratitis is produced by the combination of above 2 types of the lesion. The figure of the dendritic keratitis is produced by the characteristic nature of the herpes simplex virus wich damages the epithelial cells, along above mentioned potential channel, one by one. The part of knob (for the part of ramification) is considered as the lesion at the conflux of the channels.

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STUDIES ON THE VIABILITY OF RABBIT CORNEA STORED AT LOW TEMPERATURE

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Introduction

Recently there has been a great need for the transplantation of the cornea in the field of clinical medicine. Since 1954 when Eastcott[2] succeeded in transparant plantation of the frozen cornea in man, methods for the transplantation of the cornea have been studied from various points of view and succeeful cases in homokeratoplasty have often been reported. Now it is well known that sucess depends on the preceeding preservation of the tissue.

In order to find a suitable process of freezing for preservation it is necessary to make physiological approaches to the states of the frozen and thawed corneas. From this point of view the changes of the O_2 consumption rate were studied as reported in the present paper.

Takeuchi, one of the authors, froze eye balls of rabbits and cats in 20% glycerin-Ringer's solution at −79°C, stored them over 3 months and succeeded in the homokeratoplasty. Then he tried the plantation in man and observed that patients suffering from corneal dense opacity were markedly cured in vision by the transplantation of

the lamellar graft removed from frozen human corneas. At present penetrating keratoplasy is not successful. However, the procedure employed by Takeuchi[7] appears to be promising and it seems desirable to find out the proper conditions of this process. The medium for preservation freezing process and other procedures used in the present experiment followed mainly those described by him.

Usually Warburg's apparatus was employed for QO_2 measurement in excised tissues. The apparatus is very popular and a little practice allows one to make wide application. But it takes a long time for one determination and large quantity of a tissue is needed if the QO_2 is small. The present material, the cornea, is thin and small, showing a small value of QO_2. Moreover, as the once-frozen tissues are often rapidly and easily injured after thawing, the whole measurement in the physiological study has to be made in a short time.

In the present paper description is first presented on how the oxygraph was tested in QO_2 measurement in respect to the requirements conditions mentioned above, then the results on the frozen tissues are described.

Materials

Corneas: excised from the eyeballs of grown rabbits.

Medium for preservation: 20% glycerin-Ringer's solution. (noted G.R. hereafter)

Glycerin: 98% pure.

Medium for oxygraph: 1% NaCl solution buffered by phosphate salts to pH 7.0 (noted P.B.S. hereafter). The solution was tonometered with 54.7% O_2 gas mixture.

Methods for Low Temperature Treatments

The effects of temperature and rate of freezing upon corneal tissues were studies as follows. The excised eyeball was immersed in 5 cc G.R. in large test tubes, stored for an hour at 4°C and then slowly or rapidly frozen:

1. Removed to a thermostat of −10°C. and kept there.
2. Frozen to below −30°C. use being made of dry ice or aquid nitrogen.

Rapid freezing.

The test tube containing an eyeball was put directly into freezing mixtures.

Slow freezing.

The Rate of cooling was 1°C/min. from +4°C to −15°C, and 3°C/min from −15°C to the desired temperatures. After having been frozen and sealed tightly with gum-stoppers and vinyl paste, the tube was kept in a refrigerator of −30°C to−35°C or in an ultra-low-temperature-box. (made by Ōnishi Netsugaku Kōgyōjo Co.) of −79°C. As for below−79°C, the tube not stoppered was kept in a jar containing aquid nitrogen.

Thawing: Corneal tissue was quickly thawed by direct immersion of tube in water bath of +37° to 40°C. The material was used for test after the cornea in the eyeball or the whole eyeball had thawed. From the thawed cornea the first piece of 6 mm

diameter was cut off with catroviejo's trephine, the second and other pieces were cut round by means of iris scissors. The samples were dipped in the P.B.S. balanced with 100% O_2 gas at 37°C for five minutes and tested for determining QO_2.

Principle and Experimental Check of QO_2 Measurement by the Use of the Oxygraph

General aspects.

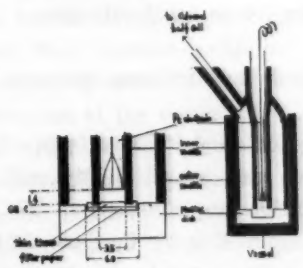
The oxygraph is a polarographic apparatus using platinum electrodes to measure O_2 concentration in liquid. The usual polarographic apparatus has wide application in the biological field. But it uses liquid electrode spoisonous to living matters. Therefore since 1950 polarographic use of platinum electrodes has been investigated. Minoshima[5] and Mochizuki[6] devised the oxygraph which charges the platinum electrode positive and negative alternatively. The negative charge causes the reduction of O_2, producing a current flow correlative with the O_2 concentration in the liquid. The positive voltage, next to the negative, is thought to activate the platinum surface and to cause the stable repetition of the O_2 -reduction.

A galvanometer is connected during the negative current phase by camswitches. The mirror deflection corresponding to the reduction, that is, to the O_2 concentration is amplified by a reflected photobeam and recorded on a photodrum. The negative charge is repeated once a quarter minute. The time interval of the negative charging is four seconds, that is, four points are plotted per minute.

The oxygraph has already found wide application.

But as the QO_2 determination in small tissues had never been tried, a special polarizing cell had to be constructed. As seen in figure 1 the cell consists of vessel and a mantle glass. The mantle glass itself consists of two mantles, the outer one is in contact with the inner wall of the outside vessel and the inner one holds the platinum electrode. A sample is laid on a small dish of aklyl resin and covered with a filter paper, which prevents the tissue from contact with the electrode surface.

Fig. 1. Schematic illustration of the polarizing cell.



Scheme of the Pt electrode Chamber Size in mm

Scheme of the electrodes Vessel

Fig. 2. Stability of the Galvanometer deflection representing the O_2 concentration in the Pt electrode chamber without O_2 consumer. One half of the whole deflection was cut off by the use of the compensator.

The vessel is filled with P.B.S. The sample is sunk into the solution, on the bottom of the vessel. The mantle glass is inserted, the inner mantle fixes the sample on the dish. The outer one holds the whole mantle glass in the most suitable position inside of the vessel. An agar bridge is inserted into the branch tube from the mantle glass, connecting the solution and the indifferent anode, calomel half cell. The platinum electrode is inserted into the inner mantle, whose tip exceeds a little the electrode surface. Between this and the tissue a small amount of P.B.S. is contained. The O_2 contained in the solution is taken up by the tissue. The current flows through the indifferent anode, tissue and platinum electrode, showing the change in O_2 concentration caused by O_2 uptake in the tissue. PO_2 in the solution at any time can be read on a calibration curve prepared beforehand.

The quantity consumed by the tissue during a time unit can be calculated. The thickness of the cornea (D) is approximately constant in adult rabbits (400μ)[9]. Assuming the dry weight to be one-fifth of wet weight and specific density of the wet weight to be 1, QO_2 could be given by

$$QO_2=\frac{\alpha \cdot 1}{D} \times \frac{\Delta p}{\Delta t} \times 18=\frac{0.023\times 0.18}{0.04} \times \frac{\Delta p}{\Delta t} \times 18$$

α: Bunsen's absorption constant of O_2 to water (0.023 at 37°C).

1: The distance from the bottom of the dish to the electrode surface (2000μ).

The thickness of the inserted filtre paper was calculated to be 200μ from the amount of displaced water. The actual value of 1 was settled to be $2000-200=1800\mu$.

p/t: The decrease in PO_2 per one time unit.

The platinum electrode employed consisted of ten 50μ pt leads fused in a glass rod.

Results.

Stability and Reproducibility. The pt electrode charged alternatively with −0.55 and +1.05 V showed a good stability as shown in figure 2, in which three sheets of filterpaper were fixed instead of the tissue sample.

O_2 consumption curve. An entire recard is shown in figure 3. The current density decreases at an almost constant rate, except for the initial few minutes, so long as O_2 is sufficiently contained in the solution. When time lapses and the PO_2 becomes low in the solution, O_2 does not diffuse to the bottom side of the tissue any longer. The O_2 consumption is suppressed. The rate of PO_2 decrease diminishes and the current density shows a gradual curve, to become linear at the PO_2 value of zero. The linear portion corresponds to the so-called residual current. In the figure the current level was raised in the couse of the decrease. The curve was once elevated uncontinously and then decreased gradually. On the left hand side the values of the current density corresponding to the PO_2, read off on the calibration curve, are plotted.

The oblique linear portion is short in the solution of low PO_2 value. The P.B.S. in the polarizing cell was equilibrated to 54.7% O_2 gas mixture previously. The samples excised in the room air are quasistationary balanced to the PO_2 of the air.

If these are subjected to measurement in this condition, O_2 in the cell solution diffuses not only according to the consumption by the tissue, but to the PO_2 gradient between the solution and the tissue. It makes the QO_2 calculation impossible. The excised samples were dipped in the solution balanced to 100% O_2 gas for five minutes at 37°C.

Fig. 3. The whole process of O_2-uptake by a piece dissected from a normal rabbit cornea.

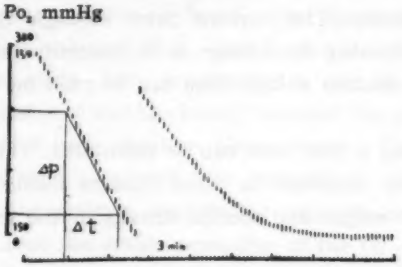
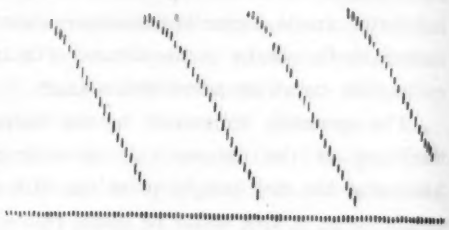


Fig. 4. Reproducibility of O_2 consumption curve by the cornea pieces dissected successively from a normal eyeball.



The good reproducibility of the O_2 consumption curves is shown in figure 4. Four samples of the cornea were taken from a normal eyeball and recorded in respect to the O_2 consumption; QO_2 was calculated to be 0.983 ($\sigma=0.036$). It took only about seven minutes to complete for one record.

Effect of insertion of filter paper. The electrode surface and the tissue are situated so closely together as to contact with each other, if the latter swells.

A sheet of filter paper is put on the tissue. When it is fixed by the end of the inner mantle, it protects the tissue from undesirable swelling and prevents contact. But then it was possible that the insertion itself could cause some effect on the current density and O_2 consumption curve. In order to check this, the sheet was laid under the tissue, and then on the tissue. No difference in current density was observed between the two cases.

The fibre of the filter paper decreases the amount of the cell solution. The water displacement by the paper was determined, subtracted from the value of 1 at the calculation of QO_2 as previously mentioned.

Conclusion.

The results described above show that QO_2 in a small amount of thin tissues is determined in a short time by the use of an oxygraph and a cell of new type.

Warburg's apparatus takes about twenty minutes after the insertion of samples for the attainment of temperature equilibrium, using large amounts of samples. It would need almost the whole cornea of one eyeball, while in the oxygraph four or five samples are obtained.

Results

QO_2 was determined of cornea with the whole layers, preserved at+4°C, -10°C,

−30°C, −79°C and −196°C for one hour to three monthes.

Preservation at +4C° and −10°C.

As shown in table 1, QO_2 was 0.913 in the tissues kept for an hour at +4°C. This value is almost equal to that of normal ones. It was 0.288 in the case of three days preservation. At −10°C after a day the cornea showed the value of 0.672, which was larger than in a cornea kept at +4°C for a day. In either case a gradual decrease was observed according to the passage of time. The freezing point of 20% glycerin-Ringer solution was about −10°C. Even after preservation, in some cases freezing did not occur in the medium.

Preservation at −30°C, −79°C and −196°C following rapid freezing.

Evers sample showed very low values. Remarkable decrease in QO_2 was found even after an hour or a day as shown in talbe 2.

Table 1. Qo_2 of corneas stored at +4°C and −10°C.

Storage Temper. (C)	Length of storage						
	1hr.	1d.	3d.	5d.	1w.	1m.	3m.
+ 4°	0.869 0.913 0.958 mean 0.913	0.378	0.288	0.164			
−10°		0.672			0.132	0.205	0.074

Table 2. Qo_2 of corneas stored at −30°C, −79°C and −196°C after quick freezing.

Storage Temper (C)	Length of stordge				
	1hr.	1d.	1w.	1m.	3m.
− 30°		0.238	0.074	0.076	0.074
− 79°	0.26	0.145	0.132	0.19	0.148
−196°	0.044	0.018			

At -30°C, -79°C and -196°C following slow freezing.

Values obtained in the samples excised immediately after thawing are shown in table 3. QO_2 at −79°C showed higher values than at −30°C. In a case of one month storage, the former was 0.359 while the latter was 0.189.

Comparison of tables 2 and 3 shows that a remarkable difference was caused by rate of freezing. In the rapid freezing 0.26 at −79°C, 0.04 at −196°C were found after an hour preservation, while in the slow freezing 0.418 and 0.312 respectively. Expected decrease in QO_2 during the period of preservation appeared clearly at −30°C but not so clearly at−79°C.

The change in QO_2 after thawing.

The first excision from the cornea was made by means of a trephine, the latter by iris scissors. The oxygraph allows only one measurement at a time. Two or more samples have to be determined successively. During the measurement of the first sample the eyeball from which the first had been removed is kept in a refrigerator. From the eyeball the second sample was excised when the first measurement was

Table 3. Qo_2 of corneas stored at —30°C, —79°C and —196°C after slow freezing.

Each value was obtained in the pieces dissected immediatly after thawing.

Storage Temper.(C)	Length of storage			
	1hr. mean	1d. mean	1w. mean	1m. mean
— 30°	0.430 0.402 0.374	0.336	0.205 0.320 0.235 0.181	0.282 0.153 0.189 0.131
— 79°	0.369 0.418 0.467	0.427 0.396 0.364	0.476 0.385 0.299	0.359 0.348 0.359 0.369
—196°	0.312	0.394		

Table 4. Post thawing injury of the corneas stored at —30°C, —79°C and —196°C.

Remarkable decrease in Qo_2 was observed as time passes.

Storge Temper. (C)	Length of storage			
	1hr.	1d.	1w.	1m.
	0.430 0.318 0.217	0.336 0.090 0.099 0.198	0.205 0.197	0.282 0.172 0.241
— 30°	0.374 0.136 0.138		0.320 0.279	0.153 0.098 0.162
			0.181 0.066 0.099	0.131 0.134 0.140
	0.467 0.171 0.157	0.427 0.382 0.213	0.476 0.189 0.057	0.359 0.100 0.123
— 79°			0.299 0.164 0.115 0.049	0.348 0.172 0.165
				0.369 0.292 0.090
—196°	0.312 0.289	0.394 0.198		

finished. It took about twenty minutes for the whole procedure of one measurement including the time for washing up the cell, drying it, balancing the sample to the high PO_2 and so on. The second sample was determined in about twenty minutes, the third forty minutes after thawing. And the remarkable decrease was found according to the lapse of time. It is shown in table 4.

In the normal cornea such a decrease was never observed. The good reproducibility in QO_2 of the normal cornea is shown in figure 4. No remarkable difference was found in the samples excised from the various parts of the cornea, that is, from the central, the upper, the lower part and so on. Trephine and scissors did not cause any changes in QO_2. Therefore the difference in QO_2 after thawing should not be considered to be associated with these things, but possibly with some physical conditions after thawing.

Check on the possible post thawing injury.

From the cornea frozen slowly and preserved at —79°C for a day, four samples were excised at one time and put into Ringer's solution, Hank's solution, own blood serum and P.B.S. After thirty minutes storage in a refrigerator at+4°C, QO_2 was determined. The sample in Ringer's solution showed higher value than the others. That suggested that the solution was good for maintenance of the activity of the cornea after thawing. From each of two eyeballs preserved at -79°C for a day after slow freezing five sam-

Table 5. Post thawing injury detected in Q_{O_2} decrease according to the lapse of time from corneas stored at —79°C for a day and kept in Rniger solution at +4°C for a few deca minutes after thawing.
The deta were obtained in the successively dissected pieces.

Times (min.) after thawing	A	B
0'	0.337	0.262*
30'	0.099	
50'	0.333	0.114
70'	0.149	0.148
90'	0.117	0.125

* +37°C. 15min in P.B.S.

ples were at one time excised. Four from amonges them were put into Ringer's solution and kept in a refrigerator for thirty minutes, while QO_2 value of one was determined without exposure to the temperature of the refrigerator. This one showed a high value. The other four showed low values as shown in table 5. The third of A in table 5 showed a high value, for which the reason remained unknown.

Discussion

The values previously reported for the QO_2 of the whole normal cornea of adult rabbits differ considerably from one another, for example, 1.44 reported by Takara[5], 0.42 by Nakamura, 0.91 by Kasahara[4], 0.55 by Duane[1] and 0.74 by de Roetth. In the present measurements the value is 0.913. The difference may be largely due to the individual difference of rabbits. According to Orzulesi the QO_2 of the cornea is inversely proportional to the body weight.

The effect of freezing on the QO_2 of the cornea was studied by Duane[1] in ox using Warbung's apparatus. In the cornea frozen rapidly to -40°C the value of QO_2 decreased to one-fifth of the normal. The slowly frozen cornea showed half of the normal value after preservation for 24 hours, and the values of one-fifth normal after four and more days. In contrast to Duane's results the cornea which was frozen slowly in this experiment showed values greater than one-fourth of the normal value even after a week. Gradual decrease in QO_2 occurred as time passed. This discrepancy might be explained by the following: (1) The difference between rabbits and oxen. (2) Duane froze and preserved the removed cornea, while the authors preserved the whole eyeball and removed the cornea after thawing. (3) In the present study glycerin was added to the freezing medium and (4) QO_2 was determined at a shor time after thawing.

In Warburg's apparatus it takes about twenty minutes are required for reaching thermoequilibrium after insertion of samples, while QO_2 decreases considerably after thawing. (see table 4). Therefore, the last reason (4) could be a cause of considerable difference.

The difference in QO_2 according to the freezing rate could be explained by the difference in the formation of ice crystals. Kanehira[3] observed trace of ice crystals in

the quickly frozen liver tissue of rats but not in the slowly frozen. The cell structure was destroyed in the former, so that the substances for respiration diffuse out into the medium solution. It resulted in the decrease of QO_2. Regarding the effect of temperature upon preservation, it was found that the cornea stored at −79°C showed values higher than those stored at other temperatures as shown in table 3.

By dipping the test tubes containing a frozen cornea into warm water of +37°C or 40°C the cornea was thawed quickly. The tissue samples were excised in some cases immediately after the thawing of the cornea and the anterior humor, in other cases after the thawing of the whole eyeball. High values of QO_2 were obtained in the former suggesting that QO_2 values were remarkably affected by the processes of thawing. In general, for biological needs, high rate of thawing may be recommended, though the exact reason is not enterely clear.

As shown in table 4 high values of QO_2 were obtained in the first samples, whilst in the second and the latter the values decreased noticeably. The second samples kept in wet air at +4°C for about twenty minutes showed the lower values than the first. This change has not been found when Warburg's apparatus was used.

In vegetable tissues a phenomenon like this was reported and named "post thawing injury". In some sorts of tissues the injury is avoided to some extent when the tissues are dipped in the solutions of special components. In the course of the present study cornea samples after thawing, were immersed in blood serum from the same animals, and also in solutions of various components. But no protective effect of either liquid was found in these cases.

The writers have expected that high values in QO_2 could always be observed in the cornea frozen slowly and preserved at -79°C, because Takeuchi had obtained good results in clinical transplantations. But high values were found only for a short time after the thawing. The transplantation is a very complicated process due to various biological mechanisms and to the necessary procedure requiring a long period. Observation focused on one of these two points should provide us with no useful information about successful transplantation, that is to say, the values of QO_2 might not show any correlation with that information. But at least, as mentioned in relation to the first samples, slow rate of freezing, rapid rate of thawing, and preservation at -79°C would be recommended.

Last it will be mentioned why glycerin was added to the medium for preservation. According to recent study on freezing, damage thereby is to some extent caused by dehydration accompanied by formation of ice crystals, perforating through cells. Addition of glycerin delays growth of ice crystals and suppresses concentration of salts resulting from dehydration. Such phenomenons produce an effective protection against the damage of cells and tissues by freezing.

Conclusion

1. QO_2 of the whole cornea of adult rabbits preserved at low temperatures was determined by the use of the oxygraph.

2. It was proved that QO_2 of small samples of thin tissue could be determined by the use of the new polarizing cell and the oxygraph. High stability and reproducibility were observed.

3. QO_2 is determined in smaller samples and in a shorter time by the oxygraph than by Warburg's apparatus.

4. QO_2 showed 0.913 average value after storage at +4°C for an hour.

5. From the values of QO_2 obtained

(a) 20% G.R. solution causes no injury to the cornea at least during one or two hours.

(b) Slow rate of freezing is dsirable.

(c) QO_2 of the cornea frozen slowly and preserved at -79°C showed comparatively higher values. That of the first piece after one month preservation was 0.359.

(d) Post-thawing injury was observed in frozen tissues.

(e) Rate of thawing had effect on QO_2 values.

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STUDIES ON AETIOLOGY AND TREATMENT OF RETINITIS PIGMENTOSA

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Retinitis pigmentosa in man is a hereditary disease of unknown aetiology which is characterized by its chronic and progressive course. Although clinically not manifest at birth, it reveals itself in childhood by the presence of night blindness, and in adolescence by the onset of retinal changes. The disease frequently results in blindness in middle or advanced life. Pathologically, this disease is essentially a degeneration of the retinal neuroepithelium, particularly of the rod cells, its established features

being an attenuation of the retinal blood vessels, optic atrophy, and almost invariably, a widespread pigmentary change in the retina.

The close similarity in morphology and the electroretinograms between the disease in man and the lesions in animals, induced by hereditarily or chemically, suggested that an investigation into the retinal metabolism in these animals might help to elucidate the aetiology of the disease, which in turn might have some bearing on the disease in man. Although the chemically induced and hereditarily induced lesions are very similar to the human disease, the pathological chemistry of the lesion in the neuroepithelium is as yet unknown.

In the preceeding paper (Mizuno, 1960) an account was given of the inhibition of the monoamine oxidase (MAO) and sulfate conjugation in the retina of a rabbit by the intravenous injection of various inducing agents. As a result of that study, it was assumed that the metabolic disturbance in the blood-retinal barrier (BRB), in which the enzymes to detoxicate catechol amine (CA) are distributed, might increase the CA content in the retina and produce impairment of the visual cells or retinitis pigmentosa.

Thus the question was raised as to whether or not some specific retinal degeneration process would be induced by a local application of CA and its metabolite. The present study has established the possibility that CA and adrenochrome (AC) destroied or disturbed the retinal metabolism and a typical retinitis pigmentosa occured.

Method and Material

The rabbits used in these experiments all were bred and maintained in the Research Laboratory of the Nagoya City University where they were kept under normal conditions and fed a complete balanced diet.

Estimation of the adrenaline (A), noradrenaline (NA) and dopa (D) contents in the retina.

The concentrations of A, NA and D in the retina, in 5 normal rabbits and 5 rabbits poisoned with sodium monoiodoacetate (MIA) from 2-7 days, was estimated. Moreover, an estimation of the A content of the retina after an intravenous injection of 0.5 ml. of 1000×A to 5 other albino rabbits was performed after mydriasis.

All procedures for the estimation of CA were performed as described by the Gamo modification (1959) of the von Euler method. It has been shown that this fluorimetric determination of each rutin is specific for A and NA and does not measures any of the other hydroxyphenyl compounds.

Intravitreous injection of CA, AC and similar substances.

Five amines in the sympathomimetica series and AC mainly were used for this experiment; however, four amines, four phenol derivatives also were used as may be seen in Table 1.

Another attempt at elucidating the nature of the action of AC was made by administering other quinones to see whether or not a similar effect could be induced in the

Table 1. Effect of various amines and phenol derivatives on the rabbit retina. These agents were dissolved in water in concentrations of 1 percent. The solutions were neutralized with bicarbonate or hydrochloric acid when they were not neutral.

Agents intravitreously injected	No. of eyes	No. of days observed	Retinal damage
1. ethyl amine	4	14	−
2. putrescine	4	14	−
3. histamine	4	14	−
4. cadaverine	4	10	−
5. serotonin	4	20	+
6. tyramine	4	10	+
7. adrenaline	10	7	++
8. noradrenaline	10	7	++
9. neosynephrine	4	10	+
10. dopa	4	11	+
11. tyrosine	2	14	+
12. caffeic acid	4	10	±
13. catechol	4	9	±
14. hydroquinone	4	10	±

Table 2. Effects of various quinones on the rabbit retina. These agents were dissolved in concentrations of 1 percent in a 0.1 M Phosphate buffer.

Agents intravitreously injected	No. of eyes	No. of days observed	Retinal damage
1. adrenochrome*1	6	20 hrs.	++
2. dopachrome*2	4	3 days	+
3. noradrenochrome*3	4	5 〃	+
4. o-benzoquinone*4	2	10 〃	±
5. β-naphthoquinone	2	3 〃	±
6. p-benzoquinone	2	10 〃	−
7. α-naphthoquinone	2	3 〃	−

*1 Prepared from 500× A by oxidation with AgO.
*2 Prepared from 500× D after irradiation with ultraviolet light.
*3 Prepared from NA by oxidation with ferricyanide.
*4 Prepared from catechol by oxidation of the catechol with Ago.

retina. The substance used, as may be seen in Table 2, were dopachrome, noradrenochrome, β-naphthoquinone, o-benzoquinone, α-naphthoquinone and p-benzoquinone. These agents were dissolved in concentration of 0.1% in 0.1 M phosphate buffer solution having a pH value of 7.5. The eyes of all the animals were washed before injection and sterilized with a 1% mercuochrome solution. Then using a 1/4" syringe about 0.3 ml of aqueous humor was withdrawn from the eye and then a volume of the

various agents equal to that of the aqueous humor withdrawn was injected into the vitreous body. To protect the test animals against possible infection they were treated with injections of penicillin. The experimental and ophthalmoscopic techniques employed were essentially those reported previously. The material was fixed in Boudin's solution and imbedded in paraffin after hydration. Serial sections were cut at 5μ, haematoxyline and eosine staining method being employed.

Treatment of experimental and human retinitis pigmentosa with new drug.

A 3 percent iodate solution was injected intravenously every day in a dose of 30 mg/Kg in 9 colored rabbits weighing about 2 Kg, 5 of which were used as the contol and 4 were injected intravenously with 2 mg of Catalin (1-hydroxy-pyrido-(3,2 a) -5-phenoxazone-3-carbonic acid) in a solution daily in additon to iodate, and effects of Catalin on the prognosis of experimental retinitis pigmentosa were observed. The experimental animals were examined ophthalmoscopically and the fundus findings of the control group was compared with those in the group treated with Catalin. The eye balls were removed on the 8th day for pathological examination.

Furthermore, an attempt was made to improve visual function of human retinitis pigmentosa with Catalin.

Visual acuity, visual field and dark adaptation were determined initially in 16 patients with retinitis pigmentosa, and Catalin injectable solution was injected subcutaneously in a dose of 2 mg for adults and 1 mg for children. It was injected daily for month initially, every other day for the following two months and 2 times weekly thereafter. During these period examinations of visual acuity with the international test chart, visual field with the Förster's campimeter and dark adaption with Nargel's adaptometer were carried out every two weeks. The treatment was continued for 44 days to one year.

Results

Contents of A, NA and D in the normal and poisoned retina.

The measurements of the A, NA and D contents in the normal and poisoned retina are shown in Fig. 1. In the retina of normal rabbits a relatively large amounts of D and relatively small amounts of A and NA were observed. However, there was a marked difference between the normal and poisoned retina, the difference being progressive up to the 5th day. On the 5th day the A and D concentrations began to decrease; however, the A concentration sometimes continued to increase up to the 7th day, and from the 2nd.-5th. day the A and NA concentrations remained very close.

Fig. 2 shows the A content of the rabbit retina before and after the intravenous injection of A. The difference in the retina between the concentration before and after the injection was only 0.0004 μg/g, while the theoretical value of A in the retina, assuming that there are no barriers and no detoxication systems in the body, is 0.24 μg/g. The results recorded in Fig. 2 confirm that no appreciable difference is

Fig. 1. Adrenaline, noradrenaline, and dopa contents of the normal rabbit retina and the one treated with iodoacetate for 2-7days.

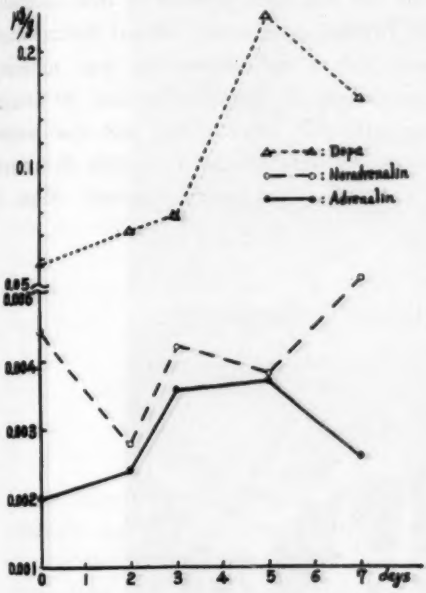
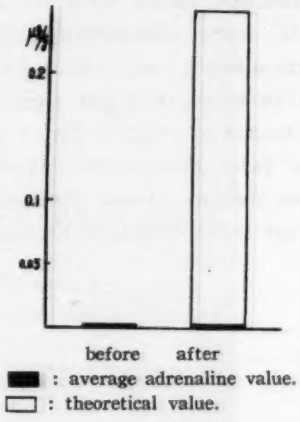


Fig. 2. Adrenaline content of the rabbit retina before and after intravenous injection of adrenaline.



present in the contents of the retina before or after the administration of A.

Experimental degeneration of the retina caused by an intravitreous injection of various agents.

The results obtained with the twenty three agents as may be seen in Tables 1 and 2, show a fairly distinct difference in their effects. Of the five sympathomimeticas, when given in a single intravitreal dose, NA and A of the diphenyl derivatives, produced a marked and rapidly developing retinal lesion similar to the retinitis pigmentosa found in humans and rabbits, while tyramine, neosynephrine, and serotonin of the monohydroxyphenyl derivatives also produced an definite result ophthalmoscopically and histologically. Of the remaining nine agents, tyrosine and D were barely positive, while the others showed only negative results.

The ophthalmoscopic picture was fairly constant with the A and NA both giving positive results, and was very similar to the one that was observed when the iodate and MIA were administered systemically. Within 2-3 days there was a loss of translucency in the retina, this being shown ophthalmoscopically by a cloudiness similating oedema of the retina, and occasionally accompanied by a slight bleeding of the retina. Pigmentary proliferation began to set in within 5 days and tended to progress for the next week. Histologically the visual cells and pigment epithelium were most affected. Fig. 3 is fairly typical of these phenomena. In contrast, the positive results obtained

with tyramine, neosynephrine, D and tyrosine were ophthalmoscopically slow at the onset, and histologically the lesion was limitted to the outer segment of the visual cells.

The most striking findings in this series were confined to AC given in a single intravitreous injection, as shown in Table 2. Of the six eyes treated in this manner with AC, three produced a marked and most rapidly developing retinal lesion, and alghouth a slight opacity of the vitreous occurred and a retinal oedema was noticed within 10 hours, a slight pigment proliferation could be observed within 20 hours which tended to progress for 1 week. Histologically the visual cells and the outer nuclear layer disappeared completly and pigment proliferation set in within 20 hours while on the other hand, the inner portion of the retina was barely affected. Figs. 4 and 5 are fairly typical of these phenomena.

Fig. 3. Marked waving of the outer nuclear layer and visual cell disappearance 6 days after intravitreous injection of noradrenaline.

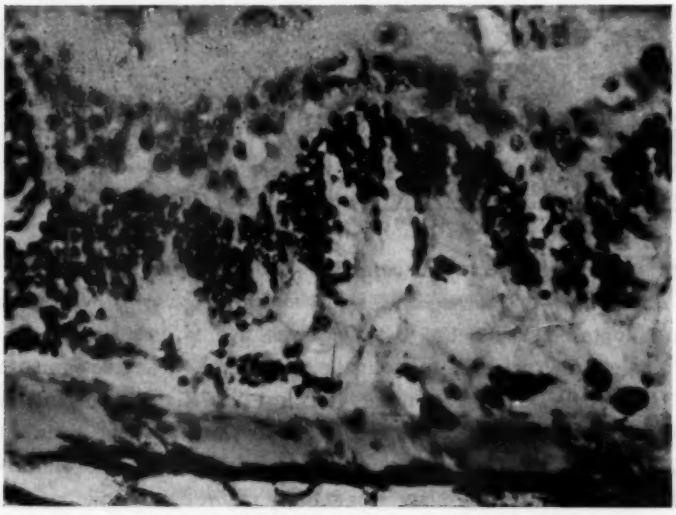


Fig. 4. Near the equator 20 hours after intravitreous injection of adrenochrome, showing complete disappearance of the outer nuclear layer and visual cell. Note abnormal pigment proliferation.

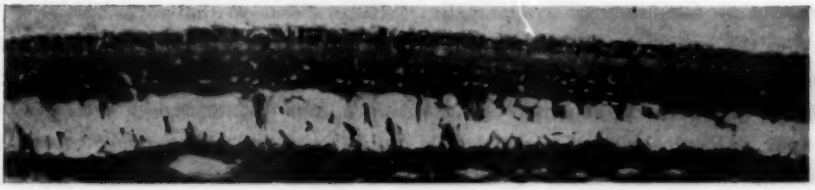
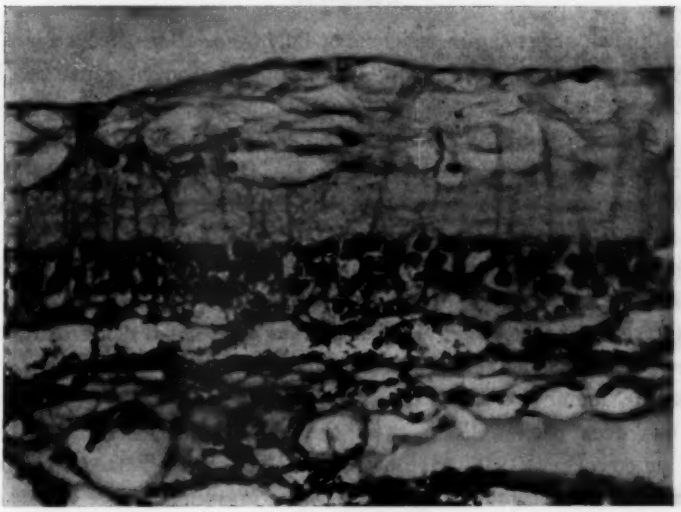


Fig. 5. Near the optic nerve 20 hours after intravitreous injection of adrenochrome, showing complete disappearace of the outer nucear layer and visual cells.



In contrast, the positive results obtained with the other quinone derivatives, for example dopachrome, β-naphthoquinone and o-benzoquinone were opthalmoscopically slow at the onset, taking a few days to show anything definite, while histologically the lesion was mild; however, with the two latter drugs, destructive appearances were observed not only in the outer portion of the retina but also in the inner portion of the retina. Two p-quinones showed nothing definite ophthalmoscopically and histologically.

Treatment of experimental and human retinitis pigmentosa with Catalin.

In the control group injected with iodate alone pigment spots developed all over the retina within 8 days on the average, while in the Catalin administered group, only a slight opacity and extremely fine pigment spots occurred in the retina and pathologically there were only disappearance of the outer segment of the visual cells and a slight proliferation of pigment on the 8th. day. These were noted on the second or third day in the control group. A marked degeneration was noted finally on 12th or 13th day.

Results of clinical application of Catalin were also satisfiable. Of 16 patients who received 16 to 176 injections of Catalin, improvement in visual acuity was noted in 11, no change in 4 and decrease in 1 case. Visual field increased in 13 and decreased in 3 cases. Dark adaptation was improved more or less in all the cases.

In the favorable cases, visual field and dark adaptation began to improve from two months after initiation of Catalin injection, and became highest after six months and the improved condition was maintained thereafter.

In the ineffective cases, cataract, optic atrophy and choroidal atrophy were com-

plicated.

Discussion

Nothing is known about the metabolism of CA in the retina except for a histochemical study by Leplat and his coworker (1956). In this present paper the quantitative analysis of A, NA and D was established. Inspection of the data presented herein shows that there is a close connection between the decrease of the MAO activity, as previously reported, and an increase of the CA activity and that during the earlier stages of experimental retinitis pigmentosa, the CA level increases to 2 times that of the normal level 2-5 days after the rabbit's retina had been poisoned with MIA. However, the concentrations of these agents were very much lower than that of the brain which previously had been reported by Vogt (1954) and Weil-Malherbe and Bone (1959). It is, therefore, possible to assume that such small concentrations of A and NA might not produce a visual cell death, but that these amines might rapidly be converted into AC under toxic conditions.

No one has ever been able to show the existance of AC in animal tissue, but Tanaka and his coworker (1954) have ascertained that the oxidized form of A, especially AC, can be an acceptor of hydrogen when in animal tissue the deamination of amino acid occurs. Mizuno (1960) had reported the possibility of an A dehydrogenase activity in a retina which had been treated with iodate.

Such a high level of CA in the poisoned retina would depend upon the malfunction of the BRB: however, these was no difference of A content before or after the systemic administration of A in the normal rabbits. This result suggests that the BRB is capable of converting the toxic amines to a non-toxic form, thus protecting the visual cells against these toxic amines.

The results recorded in this paper have introduced new agents and a completely new method for the experimental degeneration of the retina which hitherto was induced by an intravenous injection of the inducing agents. This new conception was based on the findings of metabolic disorders of CA caused by the inhibition of enzymes in the retina enabling certain toxic amines to be made non-toxic.

Thus it is very important that the experimental degeneration of the retina was induced by a metabolic intermediate such as CA or AC, because human retinitis pigmentosa is rarely caused by intoxication with drugs such as iodate or MIA.

It has been assumed that the CA will tend to concentrate in the visual cells and that it concerns the adrenergic responses as stated in the report of Leplat. Moreover, the remaining CA except the one used for nervous impluses should be destroyed rapidly within the BRB enzymatically. However, as described above an excessive highly concentrated CA may be accumulated through the inhibition or inactivation of the MAO or sulfate conjugation by systemic intoxication with various agents, or by anoxia through ligation of the ciliary artery. Therefore, from the present experiments,

it is possible to infer that the CA and AC will be selectively and specificially destructive agents to the visual cells and these substances also will be produced by the chemical or mechanical destruction of BRB.

This assumption may be supported by the fact that dopa, a structural analogue of CA, induced mild retinal degeneration, while dopachrome, β-naphthoquinone and o-benzoquinone, structual analogues of AC also induced mild degeneration of the retina rapidly, whereas the other amines, phenols and p-quinones produced no retinal damage.

In considering the relationship between the genetically induced lesions and those produced in this experiment, it is of interest to note that the distribution of the attack by AC and CA on the retina is identical to that found in sections taken from the eyes of human patients with retinitis pigmentosa. This point is further strengthened by the results reported by Walters (1959), particularly since the loss rate of anaerobic glycolysis in the retina of the rat colony is very similar to that found in studies of rabbit retina treated with MIA injections.

As discussed above, the aetiology of retinitis pigmentosa may be due to accumulation of AC or its analogues in the external layer of the retina, therefore Catalin was used in the treatment of experimental retinitis pigmentosa for the purpose of competitive detoxication of quinoids. The process of retinal degeneration could be delayed to some extent, though development of experimental degeneration could not be inhibited completely, that is, complete degeneration occurred 4-5 days later in the Catalin administered group than in the control group. Clinical application of Catalin to human retinitis pigmentosa also successful; visual field was increased, dark adaptation was improved and visual acuity was also improved though it was slight, except for advanced cases.

Catalin is a phenoxazone derivative synthesized by Ogino (1952) in order to competitively inhibit the development of quinoid cataract. The facts that retinitis pigmentosa is complicated by cataract and Catalin was effective in the patients with early retinitis pigmentosa may support this hypothesis.

Relationship between retinal metabolism and CA will be discussed in a future publication.

Conclusions

1. Chemical analysis of A, NA and D in normal retina and retina poisoned with MIA was performed. Concentrations of A, NA and D in rabbit retinas made toxic increased from 2-5 days, whereas in the normal retinas these concentrations remained the same.

2. The concentration of A in the rabbit retina before and after the intravenous injection of A showed no appreciable difference.

3. Ophthalmoscopic and histological studies on the experimental degeneration of retinas by intravitreous injection with CA, AC and their analogues were investigated. A and NA caused retinal degeneration similar to human and rabbit retinitis pigmentosa,

while dopa, tyrosine, and tyramine only caused mild retinal damage. The injury to the retina caused by CA was confined to the visual cells, the ganglion cells sometimes being simultaneously affected.

4. AC caused marked and rapid disappearance of the visual cells and the outer nuclear layer of the retina after an intravitreous injection, while dopachrome, β-naphthoquinone, and o-benzoquinone caused mild damage to the visual cells. It is suggested that CA and AC are specific and selective agents to occur the visual cell death or retinitis pigmentosa.

5. Effect of Catalin on the prognosis of experimental and human retinitis pigmentosa was observed. This new drug delayed development of experimental retinitis pigmentosa and improved visual function of human retinitis pigmentosa except for advanced cases.

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EYE-ABNORMALITIES IN MICE EMBRYOS CAUSED BY X-RADIATION OF MOTHERS —ON CHANGES IN THE INITIAL STAGE OF DEVELOPMENT BY IRRADIATION ON THE 8TH DAY OF PREGNANCY—

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Introduction

X-radiation is one of the most powerful teratogenic agents to be studied. Pagenstecher (1916)[5] subjected pregnant rabbits to x-radiation and produced rosettes in the retina of the fetus. In Japan, Yamagiwa (1936)[10] x-radiated eggs of a kind of pigeon, Streptopelia risorius Temm, and observed manifestations of typical coloboma in embryonic eyes. Russell (1950)[7] x-radiated pregnant mice at various stages of pregnancy and studied the critical period for microphthalmia, coloboma, narrow iris, open eyelid, etc. Wilson and Karr (1951)[9] observed eye-abnormalities seen in rat embryos on the 13th day of pregnancy. They x-radiated rat embryos directly under surgical operations

on the 10th day of pregnancy. Wilson, Jordan and Brent (1953)[8] also studied x-ray eye-abnormalities in rat embryos on the same developmental stage, but they treated rats on the 9th day of pregnancy. Rugh and Wolff (1955)[6] also x-radiated pregnant mice selecting a developmental stage at which the retina of embryos differentiated actively and studied the process of destruction and repair taking place in the retina.

In x-radiated embryos, developmental anomalies of the central nervous system are manifested in a high incidence. Eye-abnormalities are also often met with. These eye-abnormalities caused by x-radiation have been studied chiefly on relationship between their frequencies and the employed dose or on the morphology. Though, histopathologic studies on the process forming eye-abnormalities are only few in number. The author subjected mice to x-radiation under several steps of dosage in various stages of development and observed eye-abnormalities seen in embryos from the early stage to the later stage of development so as to study the mechanism forming the abnormality. In this paper, the author will describe frequencies and morphology of eye-abnormalities seen in mice embryos on the 13th day of pregnancy following x-radiations on the 8th day of pregnancy. The outline of the organogenesis was completed on the 13th day.

Methods and Materials

The animals used in this investigation were mice of a hybrid albino strain, and were fed with a solid diet, suppelmented by fresh greens and sufficient water.

They were about 90 days old and 22 to 26 grams in weight. Two or three estrous females were kept in a cage with a potent male overnight. Those females having vaginal plug the next morning were taken to be on the first day of pregnancy.

Four groups of mice were given a single x-radiation on the 8th day of pregnancy, the dose being 150 r, 100 r, 50 r and 25 r respectively, and each group consisting of 20 pregnant females. Embryos were delivered surgically on the 13th day of pregnancy and their entire bodies were examined under low magnification for externally visible abnormalities especially of the eye. Then, they were fixed in the Bouin's solution. The findings of the eye were studied in serial sections imbeded in paraffin, and stained with hematoxylin and eosin.

X-radiation was carried out with a therapeutic x-ray machine. The beam was that produced by the factors: 200 KVP, 20 mA, 80 cm, and the filter was 0.5 mm Al+ 0.5 mm Cu, 13.69 r/min.

A control group of 30 non-radiated females were compared with radiated ones at the same time.

Results

External appearances.

1. Embryonic deaths; as shown in Table 1, the incidence of the embryonic deaths

five days after the x-radiation revealed 41.1 per cent and 24.6 per cent, corresponding to the dose employed, i.e., 150 r and 100 r respectively. These death rates are significantly high compared with that of in controls. Death rates presented in the group treated with x-radiation of 50 r and 25 r do not have any statistical significance, when compared with that in the controls.

Table 1. Outcomes of Pregnancies of Mice X-radiated on the 8th Day of Pregnancy

X-ray dose	150 r	100 r	50 r	25 r	Controls
X-rayed mice	20	20	20	20	30
Total number of embryos	141	134	147	138	208
Embryos without abnormalities	4 (2.9%)	25 (18.4%)	101 (68.8%)	103 (74.6%)	178 (85.6%)
Abnormal embryos	79 (56.0%)	76 (56.3%)	31 (21.1%)	18 (13.0%)	9 (4.3%)
Dead embryos	58 (41.1%)	33 (24.6%)	15 (10.2%)	17 (12.3%)	21 (10.1%)

2. Number of malformed embryos were indicating those with eye-abnormalities and those with abnormalities of the brain, spinal cord, face, thorax wall, extremities etc.; in the group treated with 150 r of x-radiation, those living malformed embryos were 56.0 per cent and in that treated with 100 r, 56.3 per cent, of the total number of embryos. The manifestation rate of the malformed ones among living embryos was 95 per cent in the group x-radiated with 150 r and was 75 per cent in that x-radiated with 100 r. Though in the group x-radiated with 50 r, the rate was 24 per cent and in that with 25 r, it was 15 per cent. Thus, the rates of manifesting malformations were parallel to the doses of x-ray used. Even in the group x-radiated with 25 r, the rate was approximately three times more than in controls which showed the rate of 4.3 per cent.

Table 2. Status of Eye-abnormalities Found in Embryos Whose Mothers Were X-radiated on the 8th Day of Pregnancy

X-ray dose	150 r	100 r	50 r	25 r	Controls
Examined* embryos	83	101	132	121	187
Embryes with eye-abnormalities	21 (25.3%)	8 (7.9%)	1 (0.8%)	0	0
Unilateral microphthalmia	9	2	1	0	0
Bilateral microphthalmia	8	5	0	0	0
Unilateral anophthalmia	2	0	0	0	0
Unilateral microphthalmia with anophthalmia on the other side	2	1	0	0	0
Bilateral anophthalmia	0	0	0	0	0

*Dead embryos were excluded.

3. Excluding dead embryos, manifested eye-abnormalites are shown in Table 2. The kind of manifested eye-abnormalities presents a spectrum. The extreme case was anophthalmia in gross examinations. Those cases without eyes in gross examinations were called anophthalmias, and those with small sized eyes or vestigial signs of the eye were called microphthalmias. The above eye-abnormalities totaled 21 in the group treated with 150 r and 8 in that treated with 100 r. However, in the group treated with 50 r, there was only one case of unilateral microphthalmia and none were in the group treated with 25 r. Most of these eye-abnormalities were microphthalmias and the remaining few were anophthalmias.

Under gross examinations, 25 microphthalmias were seen on the right side and 16 cases on the left. In the experiment of the present author, a tendency was shown that microphthalmias were preponderant in number on the right side, though, a statistical significance was not evidenced.

Microscopic findings.

For reference, normal findings of the eye on the 13th embryonic day of the mouse will be stated (Fig. 1). The formation of the palpebra is started already. In the cornea-anlage, mesenchymal cells are arranged parallel to the epidermis and form a membrane with epitheliar cells. The anterior chamber is not yet formed.

The component of the lens epithel is still cylindric. The primary fibers, are arranged regularly. The cavity of the lens vesicle is almost eliminated. Though in few cases, a slight vacant space still remains.

The primordial vitreous contains capillary vessels and mesenchymal tissue. The capillary vessels reach the posterior pole of the lens associated with cells of the connective tissue. Such components cover the posterior surface of the lens and form the membrana capsularis.

The retina-anlage consists of the inner wall of the optic cup and the cells lie one upon another and, in the inner wall facing to the vitreous body, nerve fibers are found. The outer wall which will become the pigment layer is composed of a thin layer of regularly arranged cells. Both walls are usually attached to each other. he circumference of the outer wall, a vascular layer, is the presumptive choroid. The nerve fiber layer of the inner wall takes the shape of a bundle along the choroid fissure and thus forms the optic nerve.

Microscopic findings of eye-abnormalities caused by x-radiation of 150 r and 100 r were summarized as follows:

In many cases with hydrocephalus and brain hernia, various kinds of eye-abnormalities were observed. The lumen of the optic stalk was enlarged, then the formations of the optic cup and the lens were maldeveloped.

The shape of the optic cup was elongated in the direction of the optic axis (Fig. 2 and 3), or the optic cup was something like a cone and the outer wall was involved in a conspicuous infolding (Fig. 4). In such cases the lens was incompletely formed and a big cavity still remained in it.

Fig. 1. Frontal section of the right eye of a normal mouse embryo on the 13th embryonic day.

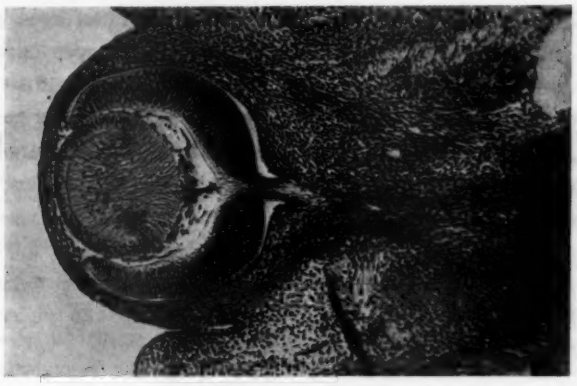


Fig. 2. X3004-$_7$: Frontal section through the right eye of a mouse embryo with eye and brain abnormalities.

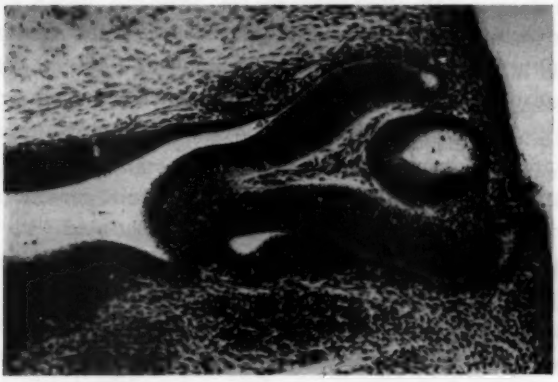


Fig. 3. X3023-$_7$: Frontal section through the right eye of a mouse embryo with eye and brain abnormalities.

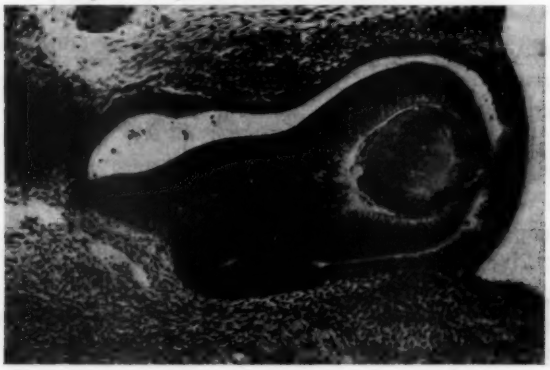


Fig. 4. X3113-$_3$: Frontal section through the right eye of a mouse embryo with eye and brain abnormalities.

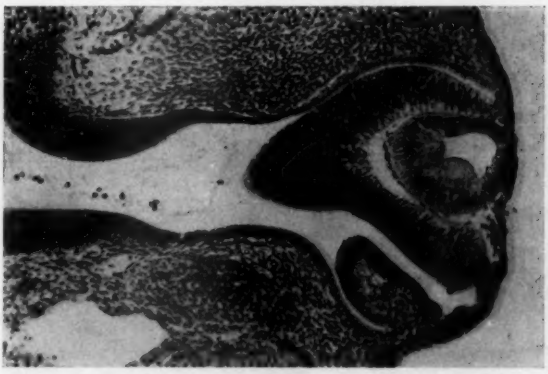


Fig. 5. X3007-$_2$: Frontal section through the left eye of a mouse embryo with eye and brain abnormalities.

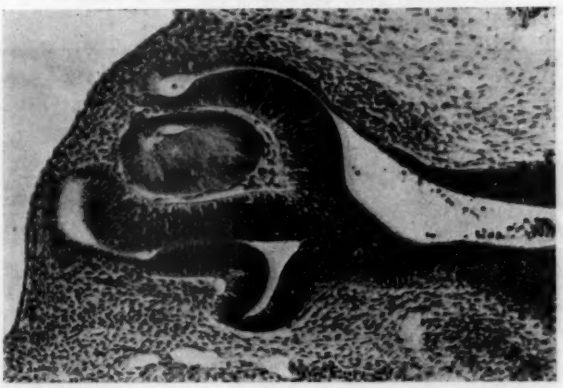
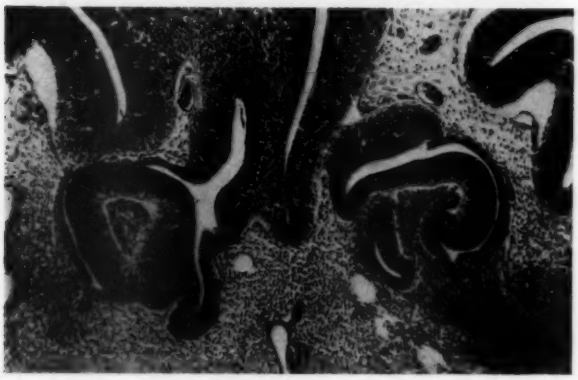


Fig. 6. X3007-$_2$: The part of the right eye of the same embryo shown in Fig. 5.

Fig. 7. X3004-$\frac{1}{3}$: Frontal section through the right eye of a mouse embryo with eye and brain abnormalities.

Fig. 8. X3021-$\frac{1}{3}$: Frontal section through the eye of the head of an exencephaly. Note localization and the direction of the axis of the optic cup.



A remarkable change seen in the optic cup was eversion of the inner wall, in the margin of the choroid fissure forming a cystic cavity by duplicated inner and outer walls. Thus, the nerve fiber was attached to the mesenchymal tissue and the choroid vessels were absent outside of the everted inner wall (Fig. 3 and 5).

Severer eye-abnormalities were the following: small sized and undifferentiated optic cups did not reach the subepidermal tissue. The lens was only rudimentally formed (Fig. 6).

In embryos with an extremely severe hydrocephalus, the above mentioned changes occurred in a higher degree. The optic stalk was almost absent, thus the posterior part of the optic cup was exposed to the 3rd brain cavity, and then the optic cup did not reach the ordinary position, the subepidermal tissue. In the outer wall, complicated infoldings were formed and young red blood cells invaded the pouch formed by infold-

ings. The lens was also absent. In the vitreous body, red blood cells and mecenchymal cells were seen (Fig. 7).

In embryos with exencephalies, the optic stalk on both sides was so rudimental that the optic cup with infoldings did not reach the subepidermal tissue. The axis of the optic cup was directed vertically instead of going to the horizontal plane. The lens was also absent and the optic cup was filled with the vitreous body having mesenchymal cells (Fig.8).

In embryos subjected to low dose x-radiations, developmental anomalies were hardly detectable even under a microscopic as well as gross examinations. Though, in embryos with retarded development the eye itself was also generally retarded. In such cases, the formation of the eye-lid was not yet started and the cavity of the lens vesicle also still remained. Among these, in some cases, the cavity of the lens vesicle occupies a considerably big space.

Comment

According to Russell (1950)[7], from the 7th to 9th embryonic day of the mouse is the critical period for micropthalmia and coloboma by x-radiation of 200 r. The present author's experiment also showed similar results employing 150 r. The anthor also found a similar relationship between eye-abnormalities employing 200 r. In the results of the present author, 100 r of x-radiation seemed to be the critical point for the rise of the manifestation rate of eye-abnormalities. One hundred and fifty r caused three times as many eye-abnormalities as 100 r. The degree of malformation also became severer. Even the deaths were probably caused by severe malformations.

King (1931)[1] stated that the hereditary anophthalmia of the mouse was detectable on the right side more than on the left side. Russell (1950)[7] also found x-ray anophthalmia of the mouse embryo predominant on the right side. In the present author's experiment, anophthalmias did not occur, but microphthalmias seemed to be more frequent on the right side than on the left side. However, it was only a tendency and no statistical significance was evidenced.

A couple of interesting findings were recognized in the present author's observations. In some cases with exencephaly or an eversion of the top of the mid-brain, a kind of cranioschisis or cerebral hernia, of which internal pressure of the brain cavity presumably escaped from the opening of the brain upward instead of going in all directions, the axis of the optic cup which was usually placed on a horizontal plane was directed vertically and the optic cup was opened in the mesenchymal tissue without the lens, and the optic stalk was also almost indistinguishable. However, in some exencephalies of which brain cavities were filled with complicated infoldings and the optic stalk seemed to be pressed by the change, the axis of the optic cup was directed in the normal way.

In many embryos with hydrocephalus, the optic stalk was also malformed and the

lumen was enlarged. In many cases with such enlargments of the lumen of the optic stalk, the optic cup was incompletely formed or malformed but was able to reach the subepidermal region. In such cases, the shape of the optic cup was often elongated in the direction of the axis of the optic cup. In the severe cases, the optic cups were not able to reach the subepidermal region. The lens formed was also small. It is not clear whether the rise of the brain pressure interferes with the normal formation of the optic stalk or not. However, it is presumable that such an abnormal shape of the optic cup is caused by the enlargement of the lumen of the optic stalk. These cases seem to be ultimately involved in a hypoplasia.

In microscopic examinations, even in the most severe malformations, the rudiment of the optic cup is usually formed. Thus, on the 13th embryonic day, there were no cases to be called primary anophthalmia[3]. Though, it is thinkable that a rudimentally formed eye on the 13th embryonic day will be eliminated in the course of the developmental stage. Hewever, a malformed optic cup as seen in the above stated exencephaly or allied abnormalities are probably caused by a general suppression or disturbance of development of the prosencephalon and will become a secondary anophthalmia[3]. Those severe eye-abnormalities such as with a rudimentally formed lens, or absence of the lens, having an enlarged lumen of the optic stalk seen in hydrocephalus, anticipates a possibility to be eliminated by degeneration and ultimately become the third type of anophthalmia, i.e., degenerative anophthalmia or consecutive anophthalmia[3].

When the above eye-abnormalities were examined under a microscope, changes in the inner and outer walls of the primordial retina were notable. The predisposed localization of many of such changes caused by eversions of the inner wall corresponded to the margin of the choroid fissure and especially in the posterior part of the optic cup. Similar findings were also presented in the process forming a hereditary uvea coloboma of the rabbit embryo studied by Koyanagi (1921)[2]. Choroid vessels were absent in the circumference of the everted inner wall and the nerve fiber of the everted inner wall was sometimes exposed directly to the mesenchymal tissue. This finding was also quite similar to that seen in the precess forming the hereditary uvea coloboma of the rabbit embryo. Such an everted inner wall, following the outer wall and the ordinary inner wall, sometimes formed a cystic cavity with fluid, and the structure seemed to press the neighbouring tissue. The wall of such a cavity was formed by the everted inner wall. Quite similar changes were also presented in the above hereditary uvea coloboma of the rabbit embryo and this change might anticipate the finding classified by Natanson[4] to the first group of the bulbuscyst or cystoma orbitopalpebrale.

Abnormalities with the same morphological patterns of hereditary disorders are manifested by some external agents. These are called "phenocopies", and the mechanism of the maldevelopment was thought to be the same notwithstanding the origine.

Thus, the teratogenic mechanism of hereditary disorders is presumed by a study on the phenocopy.

It is quite interesting that some eye-abnormalitites resulting from an external agent present quite similar findings to those seen in that of hereditary origins in their process of abnormal formation, notwithstanding the difference of employed animals, and it is worth while to study on the experimental production of abnormalities so as to solve the problem in hereditary disorders.

Summary

1. Mice were subjected to a single whole body x-radiation of 150 r, 100 r, 50 r and 25 r respectively on the 8th day of pregnancy. The embryo was observed in gross and microscopic examinations on the 13th embryonic day. Microscopic findings were emphasized.

2. Gross eye-abnormalitis were found of approximately 25 per cent in the group treated with 150 r, 8 per cent in that treated with 100 r, less than 1 per cent in that treated with 50 r and none in that treated with 25 r. All these eye-abnormalities were an- or microphthalmias. The latter were more predominant than the former in number. Almost all these were associated with abnormalities of the central nervous system.

3. In most of the embryos whose developments were retarded, development of the eye was also exceedingly retarded. Among eye-abnormalities of high degree, there were no cases classified to the primary anophthalmia, and a vestigial optic cup was evidenced under a microscope in every case with anophthalmia.

4. In hydrocephalic members, the lumen of the optic stalk was enlarged and the optic cup was also malformed in almost all cases. The shape of the eye seemed apt to become long and thin in the direction of the axis of the optic cup. In some of them the optic stalk formed was so short that the optic cup did not reach the subepidermal region and the lens was also absent. In some exencephalic members and its allied conditions, it was interesting to observe that the axis of the optic cup was directed vertically and the optic cup was opened in the mesenchymal tissue directly under the brain without having a lens.

5. In many abnormal eyes, the inner wall of the primordial retina was everted chiefly in the margin of the choroid fissure, especially in the posterior part of the optic cup. Thus, a cystic cavity was formed by such duplicated inner and outer walls. Such cases present quite similar figures to those seen in the process forming hereditary uvea coloboma and bulbuscyst. It seems to be a property of the wall pertaining to the primordial retina that it inclines to proliferate under an abnormal condition in development by either genetic or environmental cause.

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MORPHOGENESIS OF THE RETINAL CONE STUDIED WITH THE ELECTRON MICROSCOPE

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Morphogenesis and differentiation of the visual cell have previously observed by many researchers with the optic microscope, on account of its poor resolving power, each of the results is so incomplete.

Two observations recently done with the electron microscope can be attributed to De Robertis and Tokuyasu et Yamada, having considerable difference in opinion about the origin of rod sac.

Author has studied on morphogenesis and differentiation of inner and outer segment of the retinal cone in the chicken embryo, with results as follow:

Material and Method

This study was carried out on the retina of chicken embryo from the 5th day to the 21th day.

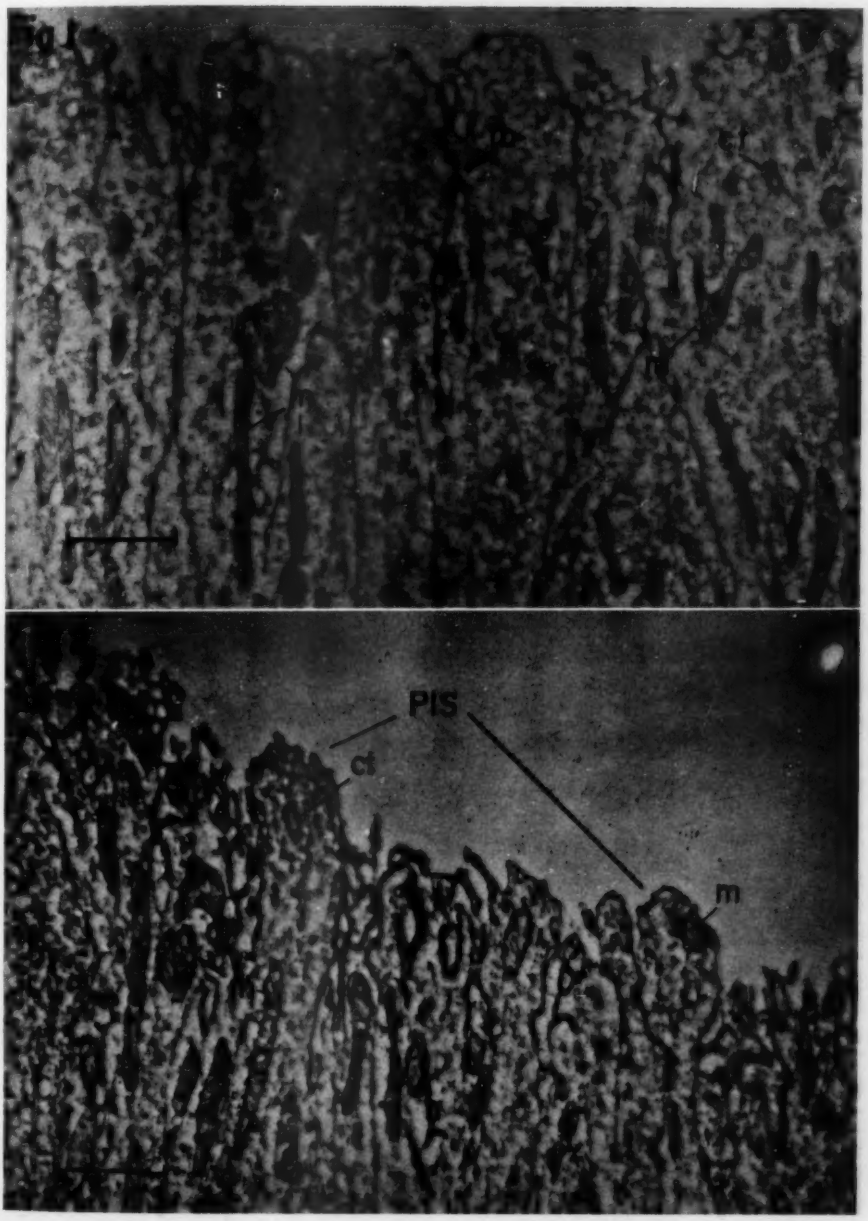
The tissues were fixed in 1% buffered osmium teroxide solution. After fixation, they were dehydrated through increasing concentrations of ethanol and embeded in n-buthyl-methacrylate: methylmethacrylate (4:1).

Sections were cut with Porter-Blum microtome and were examined mostly with a Japan Electron Optics Laboratory JEM 5C. Electron microscope.

Result

Morphogenesis of the inner segment.

In the 6th day embryo, each of the visual cells has a long axis perpendicular to the retina surface and the terminal barr is found at the distal portion of intercellular space. The distal margin of visual cells in contact with the pigment epithelium demonstrates a slight undulation of cytoplasma membrane, but there are not seen up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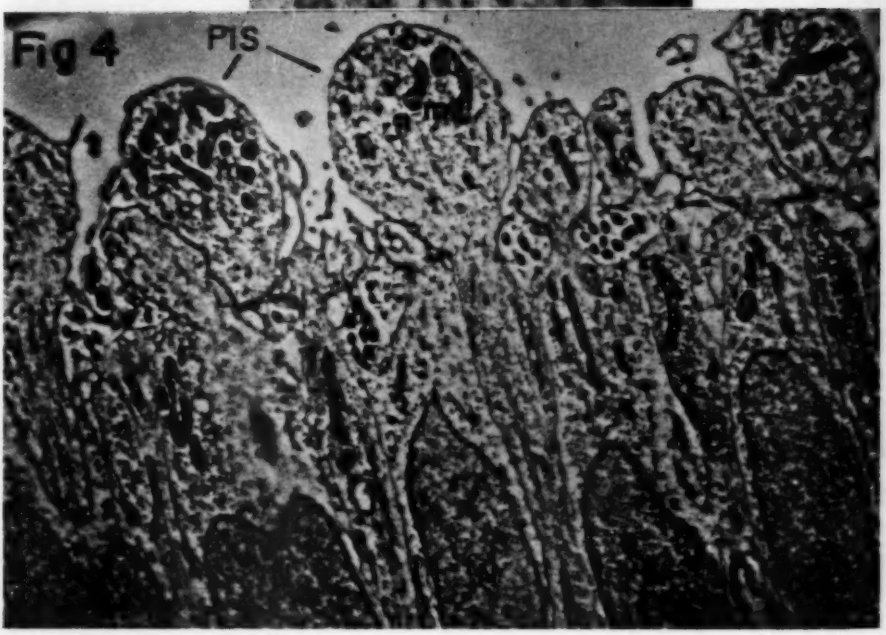
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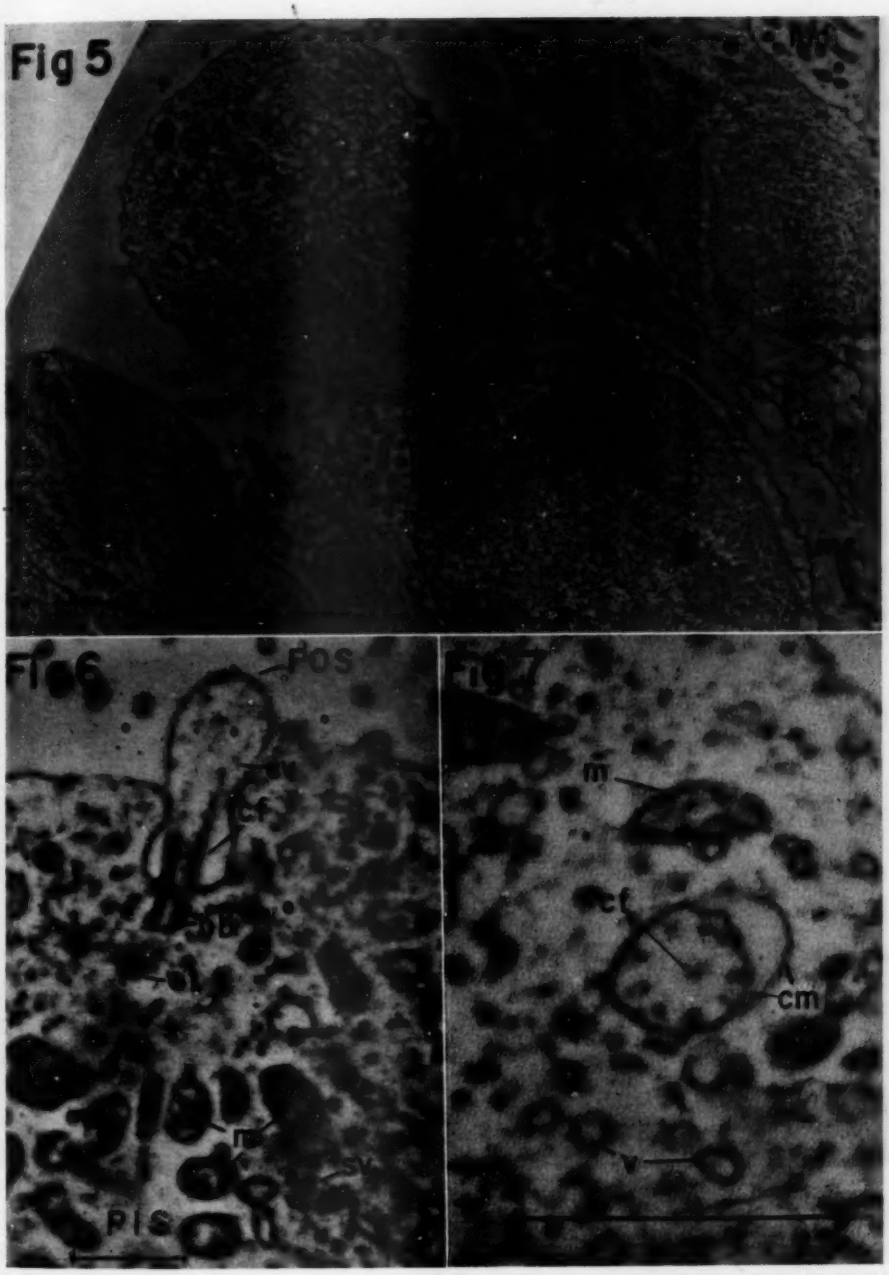
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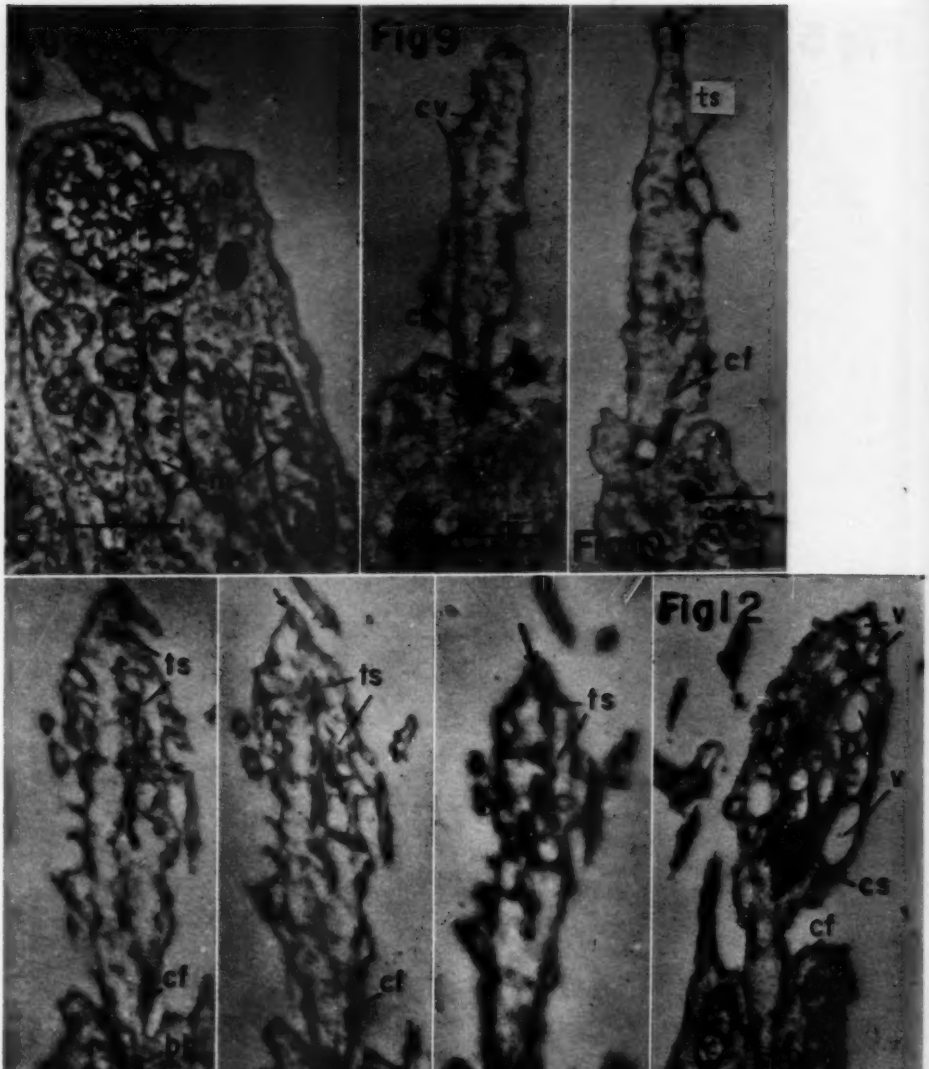
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Text Figure

Fig. 1. The distal part of the retina at the 6th day. ×16,700.
Fig. 2. The distal part of the retina at the 8th day. ×17,000.
Fig. 3. A bulge of the cytoplasma (primitive inner segment) at the 8th day. It contains small vesicles (sv), particles (sp), a centriole (ct) and mitochondria (m). The terminal barr (tb) can be seen at left of figure. ×45,000.
Fig. 4. The distal part of the retina at the 14th day. Primitive inner segments enlarge and their intracellular structure increase in number. ×7,000.
Fig. 5. The distal portion of the visual cell at the 16th day. The primitive inner segment can be seen in the top left hand of the figure, and its intracellular structure increase in number. Golgi apparatus (g) appears. ×9,000.
Fig. 6. Section through the distal portion of the inner segment at the 14th day. The primitive outer segment (POS) can be seen in upper part of the figure. ×18,000.
In the bottom of the primitive outer segment can be seen two centrioles (bb, ct), upper one (bb) projects ciliary filament (cf) into cytoplasma of outer segment. ×18,000.
Fig. 7. Cross section of the primitive outer segment at the 17th day. The primitive outer segment cut in cross section occupies the center of the field. Ciliary filaments (cf) were showing typical arrengment of nine peripheral and two central filament. ×48,000.
Fig. 8. At the 16th day, the primitive inner segment are mostly accomplished. ×20,000.
Fig. 9. The primitive outer segment at the 16th day.
At the bottom of the primitive outer segment a basal body (bb) lies and ciliary filament (cf) extend from it, then become row of small vesicles and particles (cv). ×25,000.
Fig. 10. The primitive outer segment at the 16th day.
The tubular structure (ts) can be seen in the cytoplasma, apical one connects firmly with cell membrane (arrow). ×28,000.
Fig. 11. a,b,c. The Serial section of the primitive outer segment at the 17th day.
The tubular structure can be seen. The apical portion of the cytoplasma membrane infold into the cytoplasma and connect with tubular structure (arrow). ×30,000.
Fig. 12. The vesicles become predominant in the outer segment, and in bottom the vesicles flatten and pile up. ×40,000.

and down of the cell bodies, which make all of uniform height toward the distal. As intracellular structures, a moderate amount of small vesicles and particles widely dispersed throughout the cytoplasma and mitochondria which are massive at the distal portion of cells. Besides, one or two centrioles appear at the same portion (Fig. 1).

In the 8th day one, the consequence of projecting the cytoplasma of visual cell towards pigment epithelium is that the distal surface of retina becomes so rugged. This bulge of cytoplasma is the primitive inner segment of visual cell containing one or twe centrioles, which are removed from the late position in the 6th day embryo, because one of them does not exist in the cell body, and small vesicles and particles arranged in parallel with the axis of segment (Fig. 2).

At the following stage, the primitive inner segment enlarges in fungiform and becomes to contain one or two mitochondria, whose long axis is almost perpendicular to the retina. The terminal barr is observed on the constricted cytoplasma membrane at the proximal site of this fungiform bulge (Fig. 3).

When the bulge grows more into globular and mitochondria increases in number, dense and irregular shaped structure arises within the primitive inner segment at this

periode. This is oil droplet usually found in the inner segment of cone in aves.

In embryo of the about 12 th day, the primitive inner segment grows larger in volume and its form is ovoid elongated towards the distal direction. The number of intracellular structure more increases, besides Golgi apparatus appears. The proximal part of bulge combines the cell body with the narrow cytoplasma, where small vesicles and particles are there, but no mitochondria. The neck is supported by Müller cells, which have mitochondria with a few of christa, and between Müller and visual cell, there is the terminal barr, outer limiting membrane (Fig. 5).

In the 14th day, all the intracellular structures aquire further increasing in number, besides mitochondria take irregular arrengment and various forms in C, T and V.

The primitive inner segment is further elongated to keep arrangment of mitochondria in parallel with its axis (Fig. 8).

At the following stage from the 16th day, the morphogenesis of inner segment is almost accompliated, except maturing degree by the portion in retina.

Morphogenesis of the outer segment.

In the about 14 th day embryo, it becomes possible to observe the bulge which is club-form at the distal portion of the above discribed primitive inner segment (Fig. 6). Two centrioles approch the basal portion of new bulge and the distal one (basal body) extends ciliary filaments of about 0.5μ in length into the bulge. Beyond this length, they become a row of small vesicles and particles and reach the apical cytoplasma membrane. (Fig. 9). These structure named "ciliary vesicles" by Yamada. In transversal sections, nine filaments are found in periphery—near the cytoplasma membrane which envelops the process—and two in center (Fig. 7). This bulge is the primitive outer segment.

At the following stage, some of linear structures appear in electron dense arranged two by two at intervals of about 20 mμ in parallel with each other, and completly contact with the ciliary vesicles at one edge and infrequently with apical membrane at another (Fig. 10). It is considered that the structures are tubular which are consist of electron dense membrane; serial sections make clear the observation that apical membrane is infolded into the cytoplasma of primitve outer segment and forms the tubular structures (Fig. 11 a,b,c).

The tubular structures fill at last up the primitive outer segment by numerically increasing and structurally swelling into the vesicular structures, which are round and ovoid in the sections (Fig. 12).

While these vesicular structures flatter gradually to the axis of the primitive outer segment and transform into the same to matured cone sac, they are piled up (Fig. 12) and the bulge are little widened but elongated as whole.

Desccusion

Morphogenesis of inner segment.

In spite of many theories assumed on the origin of mitochondria in the inner segment,

author has consumpted that mitochondria were not removed from the cell body but originated there in the inner segment, for the reason that there was considerable difference in the form between mitochondria of the inner segment and of the cell body, and none of mitochondria was not observed in the narrow cytoplasma, which is only one of connection.

But, it could not be made out to clarify the process of the origin.

Morphogenesis of the outer segment.

The studies on this problem have already been done with the optic microscope, but each of the results is a mere inference for its poor resolving power.

Observations with the electron microscope are attributed to De Robertis[1], who studied the development of retinal rods in the new born mouse, and Tokuyasu and Yamada[11], who investigated on the same of the rod outer segment in the new born kitten and mouse. However, on the development of rod sac is there considerable difference between them as follow: De Robertis stated, "Not ciliary filaments but vesicular structures, so called Morphogenetic Material fuse themselves and give rise to the rod sac", while Tokuyasu and Yamada described that the rod sacs were originated to the apical membrane, which was infolded into the cytoplasma by induction of ciliary filaments.

In our investigations, it was observed that the ciliary filaments which were extended from centriole into the primitive outer segment, were continuous to the apical membrane with "ciliary vesicle" in a line, but none of them could not be found, which were equivalent to the morphogenetic material photographed in De robertis' prints.

Tokuyasu and Yamada stated also that, after the ciliary vesicles made infolding of the apical membrane, the infolded membranes stretched along the ciliary vesicle and transformed into tubular structures. With the result that the infolding of the membrane, as above mentioned, was observed, author can bring himself to assert to Tokuyasu and Yamada on this subject.

The infolded membrane gives rise to the tubular and subsequently the vesicular structures, which were regarded as artifact at the time of fixation by Tokukasu and Yamada.

Of course, their opinion is probable. In observation on the cone sac in adult, however, it is considered that the infolded membrane dose not immediately become the cone sac but there are various chemical processes. Therefore, the finding that the tubullar structures transform into the vesicles is not always artifact.

Conclusion

The morphogenesis of the retinal cone has been studied with the electron microscope in the chicken embryo.

1. Observation on the morphogenesis of the inner segment.

At the distal portion of the visual cell in the 8th day embryo, the primordium of the inner segment appears as a bulge of the cytoplasma containing centrioles, small vesicles and small particles.

At the following stage, the bulge enlarges and becomes to contain mitochondria with

definite double membranes. This mitochondria dose not remove from the cellbody but arises just within the bulge of cytoplasma. This bulge is the primitve inner segment of the visual cell.

At the 15th day, the primitive inner segment elongates and increases the number of mitochondria, small vesicles and small particles. Besides Golgi apparatus appears.

And then, at the 17th day, those intracellar structures tend to assume distribution found in the adult. But the endoplasmic reticulum could not be found in all stages.

2. Observation on the morphogenesis of the outer segment.

At the 14th day, two centrioles approach apical portion of primitive inner segment, and distal one extends ciliary filaments of about 0.5 μ long in the bulge of cytoplasma of the primitive inner segment. Beyond this length, each ciliary filament becomes ciliary vesicles (named by Yamada) which pass through the matrix and reach the surface membrane of distal region of primitive outer segment.

At the 16th day, in the primitive outer segment, some of tubular structures appear which contact with the ciliary vesicle at one edge, and at another edge contact compactly with apical surface membrane. In short, the surface membrane shows deep infolding and connects with the tubular structure.

At the following stage, the tubular structure increases the number and each one enlarges laterally, and transforms into the vesiclular structure. Then the primitive outer segment is filled with numerous vesicular structures.

This structure flatters to the axis of the primitive outer segment and transforms into primitive cone sac.

Finally primitive cone sacs pile up adult disposition.

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ELECTRON MICROSCOPIC RESEARCH ON SECONDARY DEGENERATION OF THE OPTIC NERVE OF THE MOUSE

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In 1959 Yuri published results of his electron microscopic study on the optic nerve of the normal mouse. In the present investigation it was attempted to observe the ultrastructure of the optic nerve involved in secondary degeneration. That is, secondary demyelination and axon changes, which are most marked changes in optic damage, were chiefly investigated. The present investigation is to be followed by those concerning various forms of optic damage caused by experimental deficieny of vitamins, toxicosis, or allergic arachnoiditis.

Materials and Methods

Mice were used as experimental animals. The cornea was removed with a Graefe's knife from the corneal limbus, and the bulbar contents were exenterated. The excision of the optic nerve was conducted 24, and 48 hours, and 3, 4, 5, 6, 7, 10, 15, and 30 days later, and the excised nerve was fixed in osmium. Polymerization embedding was done in the ways mentioned below.

1. Embedding with Methacrylate Resin
2. Embedding with Polyester Resin

The specimen was placed for 30 minutes in each of graded aceton solutions, namely, 25%, 50%, 70%, and 90% solutions; and for one hour in 100% aceton; then twice for 30 minutes in a mixed solution containing equal parts of 100% aceton and 100% stylen; and finally twice for 30 minutes in 100% stylen. A 0.5% methyl-ethyl-keton peroxide solution and a 0.5% accelerator solution were added to a mixed solution composed of 2 parts of stylen and 8 parts of polyester (polyester: Epolac G 133 C manufactured by Nippon Shokubai Company). In the solution thus prepared polymerization was made to take place at about 20°C for 24 hours.

3. Embedding with Epoxy Resin

The specimen was dehydrated with use of the graded aceton series in the same way as described above, and then placed in a mixed solution containing equal parts of 100% aceton and araldite resin M (Ciba). After being put in resin M, the specimen was left in an incubator at 48°C for 2 hours. Polymerization was made to take place at 48°C for 48 hours in a mixed solution containing resin M, Hardner 964, dibutylphtalate, and Accelerator 964 at the rate of 100 : 100 : 10 : 3. Before embedding the central and peripheral parts of the optic nerve were respectively marked so as to facilitate the identification of the direction of the optic nerve at the time of preparing sections. In order to fix a

Fig. 1. A cross section through the normal optic nerve of mouse with use of prepolymer polymerization embedding. ×50,000

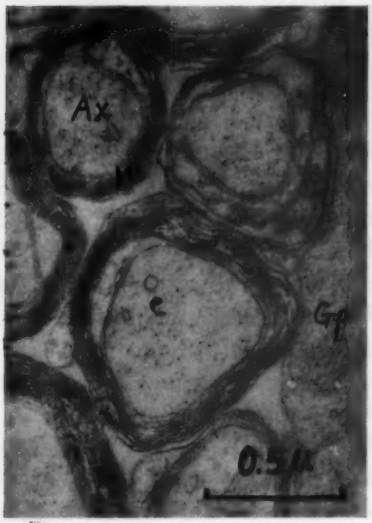


Fig. 2. Same to Fig. 1. ×35,000

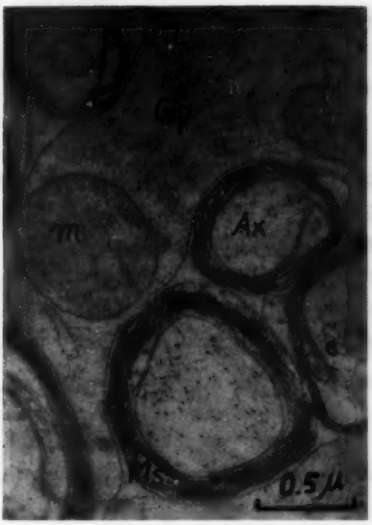


Fig. 3. A cross section through the normal optic nerve of mouse with use of methacrylate polymerization embedding. Not to artificial separation and fragmentation of the myelin sheath. ×13,000

Fig. 4. Same to Fig. 3. ×5,000

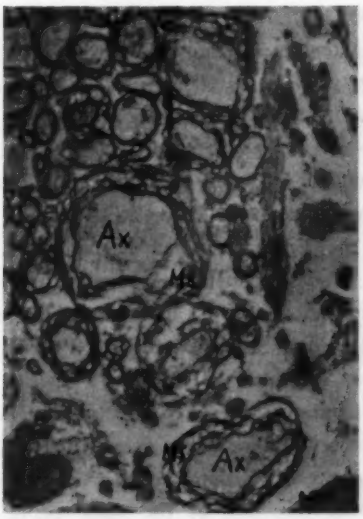
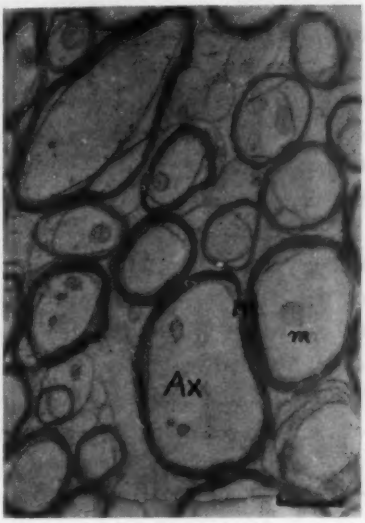


Fig. 5. 24 hours after the surgical procedure no changes are observed. ×12,000



site to be observed, the length of the optic nerve from its bulbar attachment to the chiasm was trisected, and a one-third portion before the chiasm was used for observation. A Tronscope 50 (Akashi Company) was used for electron microscopy. A 5G-type electron microscope (Nippon Densi-Kogaku Company) was also used in some cases.

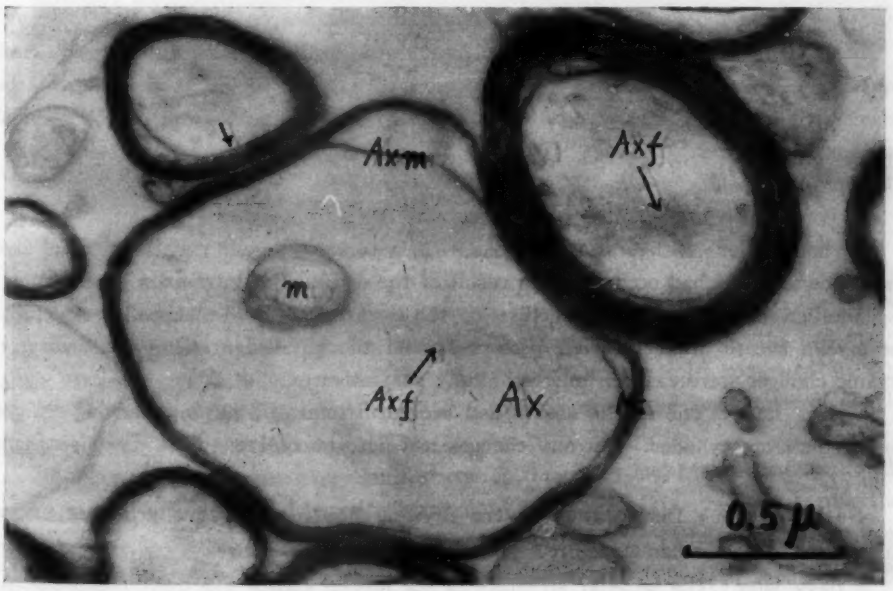
Results

The cross sections of the optic nerve before the chiasm were chiefly used for observation.

Changes in the axon cylinder.

Twenty-four hours after the surgical procedure no changes were noted in the internal structure of the axon cylinder, namely, in endoplasmic reticuli, mitochondria, and axon filaments. In a few

Fig. 6. 48 hours later: Changes of some degree have appeared. The lamellar structure of the myelin sheath is still well preserved. ×50,000



nerve fibers, however, mild depression of the axon membrane was present (Fig. 5).

48 hours later: Changes of some degree had appeared in all myelinated and non-myelinated fibers. Swelling, and sometimes mild disintegration of endoplasmic reticuli were noted. Mitochondria, too, showed mild enlargement and swelling, and tended to gather round the axon cylinder (Fig. 6).

3 days later: Endoplasmic reticuli were broken into fine granulelike fragments, and these fragments had formed a cluster round the axon cylinder. Where endoplasmic reticuli, falling into disintegration, had finally disappeared, the axon cylinder showed a hyalinized internal structure, and accordingly a very low electron density. Mitochondria, too, were markedly swollen and their density was heightened with blurring of their cristae (Fig. 7). The axon filament, normally dotlike in a cross section, presented a netlike appearance owing to advancing degeneration. The axon cylinder, too, which is normally circular in a cross section, was gradually disfigured owing to the decrease of intraaxon tension, as the disintegration and disappearance of its internal structure went on.

4 days later: Endoplasmic reticuli, mitochondria, and axon filaments, falling into disintegration, had disappeared. And so the inside of the axon cylinder assumed a lighter tone, and uniformly showed a lowered density. But in some places the density was heightened owing to condensation of disintegrated fragments (Fig. 8).

5 days later: In large myelinated fibers the internal structure of the axon cylinder had suffered disfiguration and atrophy, and it was rather difficult to identify the axon cylinder. Myelin sheaths and small myelinated fibers, too, were either markedly or completely disfigured. The inside of the axon cylinder showed a uniform increase of density, and the observation of its minute structure was impossible.

10-15 days later: Disintegrated myelin sheaths had clustered and formed massive lumps. The axon cylinder, too, being covered with these lumps, had here and there degenerated into dense masses. Its structure being thus quite obscured, the axon cylinder was hardly recognizable as such (Fig. 14, 15).

Changes in the myelin sheath.

24 hours later: No changes were observed.

48 hours later: Disfiguration was observed in some of myelin sheaths of large myelinated fibers. This had occurred as a result of the decrease of intra-axon tension due to degeneration of the internal structure of the axon cylinder. The lamellar structure of the myelin sheath was still well preserved, and though disfiguration was present, no organic changes were electron microscopically demonstrated (Fig. 6).

3 days later: The myelin sheath had become flattened owing to advancing disfiguration of the axon cylinder. But any changes had not yet occurred in the lamellar structure (Fig. 7).

4-6 days later: Disfiguration of the myelin sheath had gradually become marked, and with it, the lamellar structure had lost its original distinctness.

7 days later: The lamellar structure of the myelin sheath was quite obscured. And

Fig. 7. 3 days later: Mitochondria and endoplasmic reticuli are markedly swollen. ×22,000

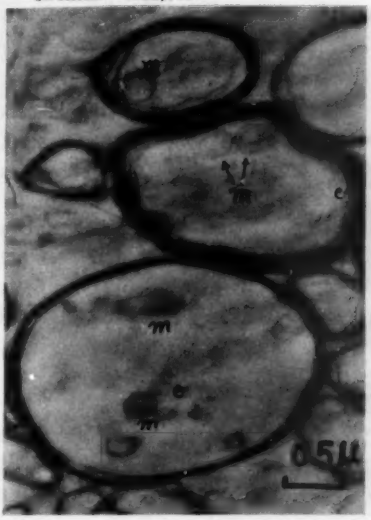


Fig. 8. 4 days later: Endoplasmic reticuli, mitochondria, and axon filaments, falling into disintegration, have disappeared. Disfiguration of the myelin sheath have gradually become marked. ×32,000

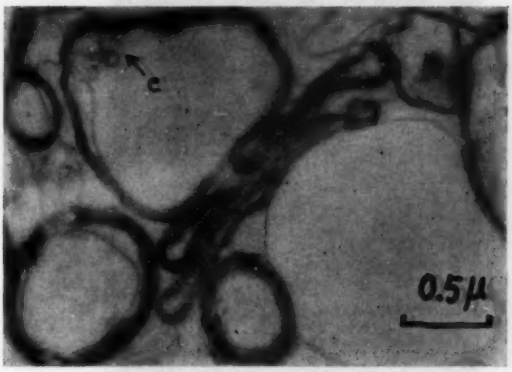
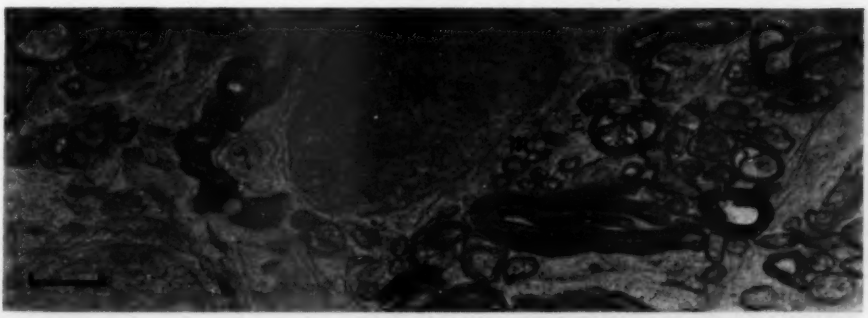


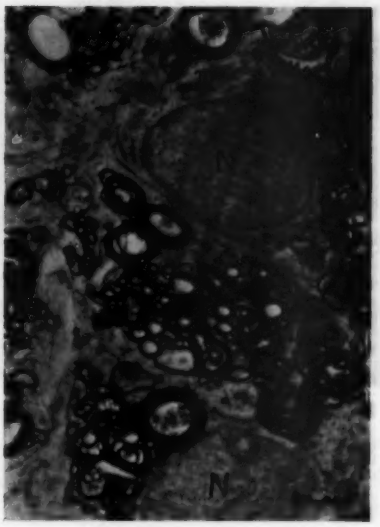
Fig. 9. 7 days later: The myelin sheath have become flattened. ×12,000



with atrophy and shrinking of the axon cylinder the myelin sheath had become flattened, being oppressed by the surrounding tissues. The flattened myelin sheaths had encircled other flattened myelin sheaths, and thus two or three sheaths were combined in layers. As their tips were joined together, they sometimes presented the appearance of double or treble concentric circles (Fig. 9,10,11).

10 days later: Almost every unit of concentric circles had become a massive lump; and not only myelin sheaths but also nerve hard to identify (Fig. 14). It seems that a group of myelin sheaths connected with a glial membrane come to be subjected to pressure as a unit with advancing shrinking of the axon cylinder and proliferation of the sustenta-

Fig. 10. 7 days later: Endoplasmic reticuli in glia cells show a tendency towards flattening and hypertrophy. ×7,500

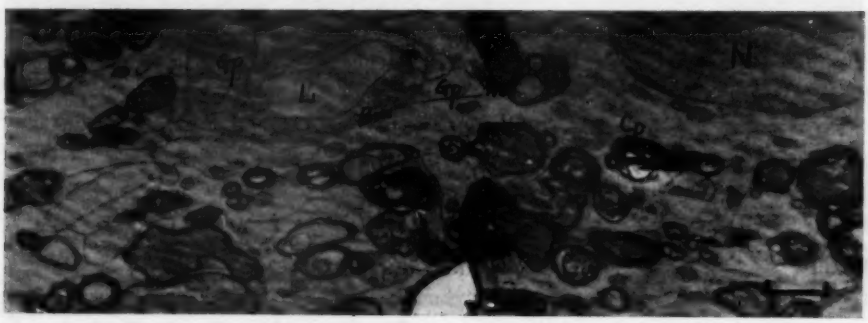


cular tissues; and that small myelinated fibers tend to gather round gradually-flattened large ones (Fig. 13). Large fibers were severely affected by these pathologic changes already in the early stage, but small ones remained resistant for a considerably long time. This fact has not till so far been reported in any previous papers. Previously reported fragmentation, separation and vacuolar formation between myelin-sheath lamellae was not met with in the present investigation. Therefore such findings as were obtained by Roizin, Omi and others may be considered to be artifacts.

Glia cells.

2-3 days later: Degeneration of the axon cylinder was seen to be accompanied by disfiguration of large myelinated fibers. Proliferation of glial processes was at the same time noted.

Fig. 11. 7 days later: Capillaries are encircled all round by glial processes. ×10,000



4 days later: Glia cells showed proliferation and hypertrophy, and because of this large myelinated fibers suffered compression, flattening and atrophy. And the space produced by the atrophy had been nearly entirely filled up by glia cells. During this phase endoplasmic reticuli in glia cells were generally not so markedly disfigured, but showed a tendency towards flattening and hypertrophy (Fig. 10). As for mitochondria, they, being swollen, were present in large numbers in the periphery of the cytoplasm Golgi's apparatus had become distinct, and granules had increased in number.

Fig. 12. 7 days later: A glia cell of the 1st type. ×7,500

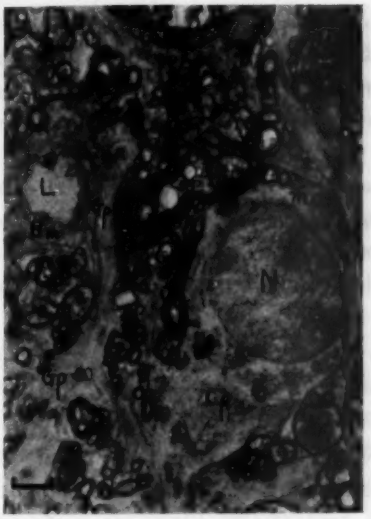


Fig. 13. 15 days later: Small myelinated fibers tend to gather round gradually-flattened large ones. ×22,000

Fig. 14. 15 days later: Almost every unit of concentric circles have become a massive lump. ×13,000

Fig. 15. 30 days later: Disintegrated myelin sheath have clustered and formed massive lumps. ×21,000

10 days later: The density of the cell was uniformly heightened. The boundary line between the nucleus and the cell membrane had become blurred, and the unevenness of the nuclear membrane prominent.

After 15 days later: The cytoplasm had markedly increased in volume. Nerve fibers surrounded by a glia cell showed a definite tendency to grouping, and, having fallen into atrophy and degeneration, were localized into a colony amid glia cells (Fig. 13).

Blood vessels and connective tissue.

Capillaries were encircled all round by glial processes. In Fig. 11 which is a picture taken 7 days later pericapillary nerve fibers are seen to have degenerated, shrunk, and been disfigured. Proliferation of glia cells accompanying the decrease of nerve fibers is also observable. But perivascular nerve fibers do not show particularly conspicuous degeneration, compared with the same elsewhere.

Discussion

Polymerization embedding.

The preparation of material from central nerve fibers demands some consideration; many investigators have noted that such artifacts as separation and fragmentation of the myelin sheath are usually produced even in normal material during the process of preparation. Yuri and associates have long sought to obviate this defect by technical improvement, and tried to ascertain what process of preparation is responsible for the production of such artifacts by conducting basic investigations on methods of fixation, buffer solutions, methods of taking material, and polymerization temperature. And they have found out that the production of such artifacts is inevitable, as long as methacrylate is used. That is, material and method being the same, mild disintegration of the myelin sheath was noted in the case of methacrylate being used, whereas polyester showed no production of any artifacts. It was thus ascertained that separation and fragmentation of the myelin sheath occurred during the process of polymerization embedding; and therefore that the production of artifacts was due to greater compression, and higher momentary polymerization heat caused by methacrylate. Such being the case, polymerization with methacrylate resin is considered unsuitable for the investigation of pathologic nerve fibers, especially of myelin sheaths. That Omi and Roizin made wrong observations, the former in his investigation on peripheral secondary degeneration, and the latter in his investigation on experimental allergic meningitis is due to their paying no attention to this fact. The present authors believe that their close scrutiny into such a basic experimental condition will contribute much to the progress of electron microscopy, and at the same time enhance the significance of the present investigation.

Changes in the axon cylinder.

Using peripheral nerves, Honjin, Vial and Omi studied the early phase of Waller degeneration in the axon cylinder. According to Honjin who used the sciatic nerves of the frog and the mouse, the axon cylinder is first affected, and then 36 hours after severance

changes appear in the endoplasmic reticuli of large myelinated fibers. As for mitochondria, they fall into complete disintegration 6 days later, and as to axon filaments, the disturbance of their longitudinal course is noted 48 hours later, and 6 days later they show a complete disintegration. Vial noted that endoplasmic reticuli lost their normal shapes 24 hours later in secondary degeneration of the sciatic nerve, and further demonstrated swelling of mitochondria by measurement, though in the opinion of the present authors mitochondrial swelling is better judged by disfiguration than by measurement. He also stated that axon filaments showed rather advanced disintegration already 24-48 hours later. According to Omi, swelling and disintegration of endoplasmic reticuli and mitochondria commence 24-48 hours after severance in secondary degeneration of the sciatic nerve of the rabbit. The present authors, using mice, noted that intra-axon depression of the axon membrane was present 24 hours after the surgical procedure, though no changes were observed in the internal strcuture of the axon cylinder, namely, in endoplasmic reticuli, mitochondria and axon filaments. This finding obtained by the present authors differ fundamentally from those by the above-mentioned three investigators. And it seems impossible to attribute this difference solely to differences between the peripheral and the optic nerve. This finding is considered a first sign of disfiguration of nerve fibers which occur owing to the decrease of intra-axon tension. Four days later the internal structure of the axon cylinder falls into complete degeneration.

Honjin stated that large myelinated fibers were sooner affected by degeneration than small ones, but Omi expressed a contrary opinion. In the present investigation large myelinated fibers were clearly affected in an earlier stage. This fact, together with peculiar changes of the myelin sheath, deserves a special notice. In large myelinated fibers the internal structure of the axon cylinder is affected 48 hours after the surgical procedure; that is, the chemical decomposition of axon componets causes the decrease of intra-axon tension, and compression of the axon cylinder. And then the nerve fibers of small myelinated fibers which are in contact with large ones are disfigured, and with the passage of time, gradually flattened and compressed.

Changes in the myelin sheath.

With atrophy and disappearnce of the axon cylinder the myelin sheath comes to be compressed owing to proliferation of adjacent glia cells, and finally presents a peculiar disfiguration and degeneration. The process of its degeneration deserves a special notice, as it has not yet been properly described by other investigators.

Electron microscopic observation of degeneration of the myelin sheath has so far been done by two investigators: Omi who studied secondary degeneration of peripheral nerves, and Roizin who investigated experimental allergic meningitis of the guinea pig. The findings obtained from the optic nerve may as well somewhat differ from those obtained from the sciatic nerve and the spine, but the above-mentioned investigators commited a fatal mistake by describing as pathologic those artifacts which are often produced in the myelin sheath of the normal optic nerve of the mouse when methacrylate is used for

polymerization. These artifacts are very similar in appearance to the so-called separation, fragmentation or disintegration of the myelin sheath. The present authors, by comparing the results obtained by the improved method of polymerization with those by the traditional ones, have arrived at the conclusion that the peculiar degenerative process of the myelin sheath ascertained in the present investigation is not restricted to the optic nerve of the mouse, but common to all nerve fibers generally. That is, degeneration of the axon cylinder of the large myelinated fiber is accompanied by the decrease of intra-axon tension, and this decrease in turn causes compression of the axon cylinder, and finally as a result of it, disfiguration of the myelin sheath occurs 48 hours after the surgical procedure; but still at this time the lamellar structure of the myelin sheath is well preserved, and the myelin sheath itself remains unaffected. As mentioned already, this fact shows that degeneration of the axon cylinder preceds that of the myelin sheath.

From 3 days later onwards the myelin sheath becomes more and more disfigured with advancing degeneration of the axon cylinder, and being gradually flattened, shows a cyclical winding. Intact, relatively thin myelinated fibers frequently gather towards large ones. After 6–7 days later the lamellar structure of the myelin sheath loses its original distinctness, and about 10 large and small myelin sheaths as a unit show a tendency to grouping. These sheaths seem to originate from one glial membrane. Two or three flattened large myelinated fibers encircle one middle-sized one, and as their tips are joined together, they form a myelin-sheath ring which presents the appearance of double or fivefold concentric circles. Ten or 15 days later these myelin sheaths concentrically arranged are fused into one dense mass.

Changes in the glia cell.

From 4 days later endoplasmic reticuli in the cell which may be regarded as an oligocyte become flattened and slender. Mitochondria, too, show a tendency to mild enlargement. From 7 days later granule substance in the cell increase in quantity, and mitochondria are definitely enlarged. Ten or 15 days later the cells generally show a heightened density, and the nuclear membrane becomes markedly irregular. This phenomenon is said to have something to do with the activity of the glia cell. It is said that in peripheral nerves phagocytes clear away disintegrated masses of myelin sheaths, but in the present investigation the process of phagocytosis was not sufficiently observed.

Blood vessels and connective tissues.

Primary demyelination is said first to occur around blood vessels, but in secondary degeneation of the optic nerve blood vessels seemed to have no special significance. Marked proliferation of glia cells, however, was noted around capillaries where nerve substance was reduced in quantity owing to degeneration and contraction of nerve fibers. Much is left for future investigation concerning the behavior of connective tissue cells and perivascular glia cells.

Conclusions

Electron microscopic observation was conducted on secondary degeneration of the

optic nerve of the mouse with use of prepolymer (polyester, epoxy resin) polymerization embedding. Findings obtained are as follows.

1. Separation and fragmentation of the myelin sheath electron microscopically demonstrated in the past are considered to be artifacts produced during the process of polymerization embedding.

2. No production of artifacts is noted when the prepolymer preparation is used for polymerization embedding.

3. A first sign of secondary optic degeneration appears in endoplasmic reticuli in the axon cylinder 48 hours after the surgical procedure, and then mitochondria and axon filaments are affected in the order mentioned.

4. Large myelinated fibers are affected earlier than small ones.

5. The internal structure of the axon cylinder falls into complete disintegration after 5 days later.

6. The lamellar structure of the myelin sheath is still well preserved 48 hours later.

7. Six or 7 days later the lamellar structure of the myelin sheath loses its distinctness, and becomes obscured. The tips of flattened myelin sheaths are joined together, and these sheaths form a concentric ring.

8. Ten or 15 days later this concentric ring turns into a large dense mass.

9. After 7 lays later glia cells show a gradual tendency towards proliferation.

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A STUDY ON THE MEASUREMENT OF OCULAR AXIAL LENGTH BY ULTRASONIC ECHOGRAPHY

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Oksala A. and Lehtinen A[3]. succeeded in detecting intra-ocular foreign bodies, detachment of the retina and rupture of the sclera by means of ultrasounds, and furthermore, measured the velocity of utrasounds in parts of the eye. As this fact suggested the possibility of measurement of ocular axial length, the authors have succeeded in the measurement of ocular axial length by a pulsed ultrasonic echographic equipment in cooperation with vice-president & professor Iwatake M. and lecturer Ishida M. at the Shibaura Institute of Technology, Tokyo, Japan. Results obtained were already partially published at the general meeting of Tokyo Chapter of Electricity Society held in November, 1959, but further researches on this topic will be reported as belows.

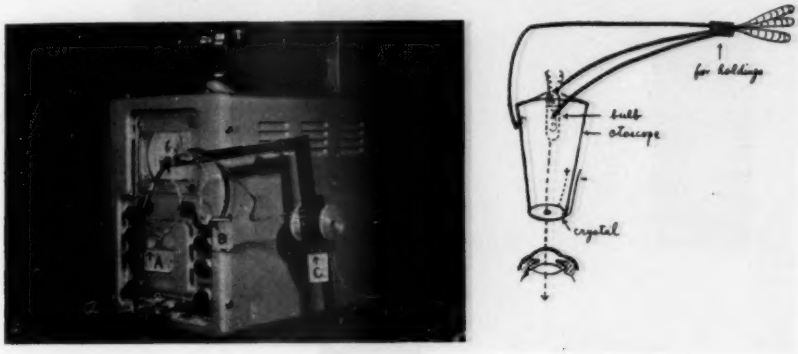
Fundamental Experiment

The crystal frequency, 5 mm ϕ, 5 Mc, was chiefly used as the searching unit by the use of an equipment made by the Shibaura Institute of Technology. A semi-transparent part of about 1 mm was provided at the centre of crystal frequency. An aural speculum affixed with the crystal at the tip and furnished with the bulb at the fundus was used as the searching equipment. This was fixed to the traveling apparatus. At hand manipulation, on the other hand, the handgrips of three cotton wound applicators equiped at the fundus of otoscope as shown at the right side of Fig. 1. was put between inspector's fingers, the crystal contacting tangentially with cornea of the examined eye. Then the examined eye gazes at the filament of bulb through the semi-transparent part of crystal. As a result ultrasounds emitted from the crystal reach exactly the macula lutea of eye fundus. Pressure of the searching equipment upon the cornea in the course of measurement disturbs the picture of filament recognized by the examined eye, so that caution is taken.

When the crystal is stuck to one side of micrometer, and echograph of ultrasounds in water is measured by 5 inches Braun tube at the other side, the distance between both sides and that between discharging and receiving echographs on the Braun tube are so completely placed on the straight line that the greatest horizontal amplitude of Braun tube, which corresponds to 30 mm in the measurement of ocular axial length, is kept in a certain condition.

Next, as to the orientation of crystal, the theoretical value[2] is indicated by the formula

Fig. 1. The ultrasonic equipment and the searching unit at the tip of otoscope. The hand fixing apparatus utilizing three cotton wound applicators is indicated at the right side.
Ultrasonic eqwpment
A searching unit
B bulb
C traveling apparatus



$\theta^0=\sin^{-1}0.61\frac{\lambda}{R}$ (λ means $\frac{\text{velocity (cm)}}{\text{cycle}}$, and R signifies radium (cm) of the crystal). There are noted different orientations in crystals practically used, and the measurement demonstrates that at the distance of 23 mm in water 3°44′ (theoretical value 2°33′) and nearly 5° (theoretical value 4°5′) orientations are noted in crystals of 8 mm ϕ, 5 MC and 5 mm ϕ, 5 MC respectively. If applied to the eye fundus almost no crystal is emitted to any region other than the macula lutea. Accordingly, the echography is possible only in the region of macula lutea, and conforms to the measurement of ocular axis.

Next, the origin of echograph is involved. Bovine sclera of about 2 mm width is first drawn at the centre of a small box as shown at the left side of Fig. 2 and divides the box into two sections A and B. Each section is filled with various substances as shown in the figure, and whenever ultrasounds are emitted, echoes are received at the front of bovine sclera. In case of human eye air is filled into Tenon's capsule as indicated at the right side of Fig. 5. This is intended for causing the generating point of echo to move foreward by the filling of air having the greatest capacity to intercept ultrasounds, if the generating point is situated in the tissue backward of the eyeball. However, the result is much the same. Thus, the generating point of echo is concluded to be noted at the inner wall of eyeball, perhaps at the front of retina. On the other hand, we measured the velocity in the eye partially using the crystal stuck to the micrometer, i,e, sclera (bovine) 1.66×10^5 cm/s, cornea (pig) 1.58, lens (pig) 1.53, lens (human, cataract) 1.57, both acqueous and vitreous (bovine) 1.47, consequently, the average velocity in the eye from Gullstrand's informal eye pattern was calculated at 1.48×10^5 cm/sec. In these determinations, the velocity of water at 4°C 1.43×10^5 cm/sec was adopted as a standard.

Fig. 2. The origin of echograph is investigated. On the experiment, bovine sclera as shown at the left side and human eye as shown at the right side is in use respectively.

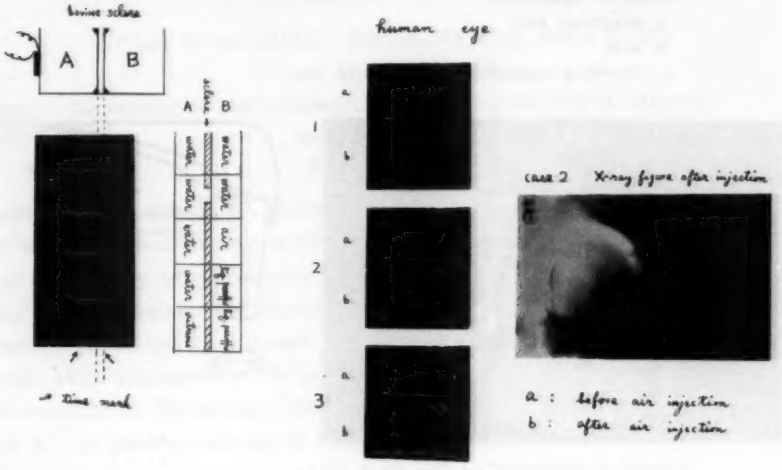


Fig. 3. The conversion table to the ocular axial length from the generating distance of echo reproduced on 35 mm film from the picture in Braun tube is shown.

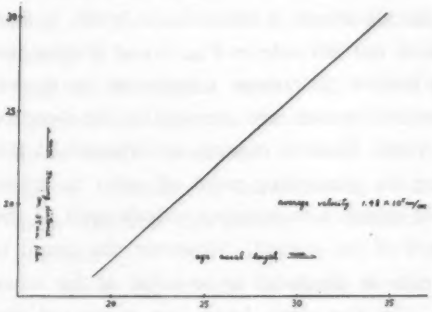


Fig. 3 indicates that the picture in Braun tube is reproduced on 35 mm film, and the ocular axial length is calculated from the measurement value of the reproduced picture.

Clinical Experiment

There are two considerations as to the measurement error. The one is concerned with the equipment, while the other is concerned with the technique. As far as the equipment is concerned, it depends on the non-uniformity in the make of used crystals and variation in their amplitude. As ample attention is taken to these points, there is no danger of error being introduced in the measurement. As to error involved in the technique, no error is liable to be introduced when the travelling apparatus is

Fig. 4. This photograph demonstrates the measurement by hand is sufficiently correct.

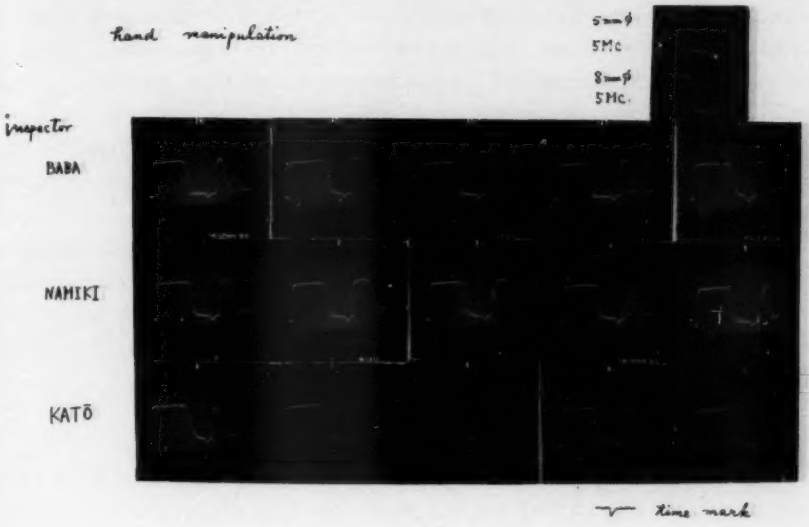
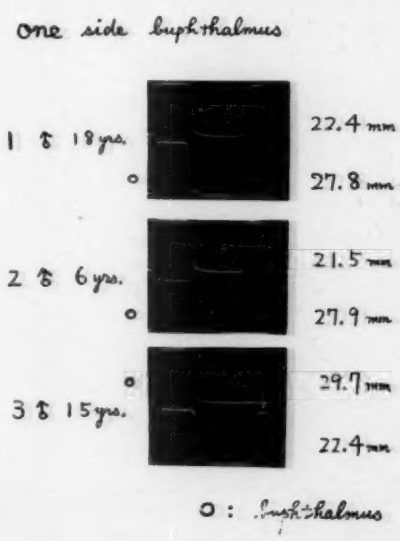


Fig. 5. The relative echograph between normal eye and buphthalmus is demonstrated.



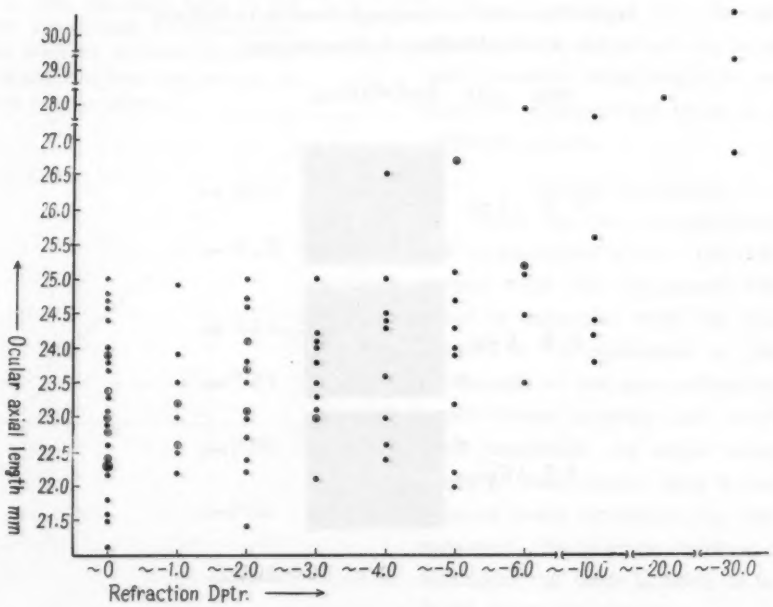
resorted to, but there is danger of error being done in hand fixing of the crystal. When a special kind of fixing equipment using cotton wound applicators is taken into consideration, errors are unexpectedly introduced into the echograpy on account of 5 seconds less of its contact with the cornea. Fig. 4 indicates that the measurement by hand was repeated on the same examined eye 5 times respectively by three inspectors. Generating distance of echo is almost the same in every case. In this case gazing at the Braun tube and filming of the picture were entrusted to unexperienced nurses in order to keep off preoccupation. The inspectors had nothing to do with the process. This fact demonstrates the measurement by hand to be sufficiently correct.

Thus, echographs over emmetropia, anomalies of refraction, buphthalmus (Fig.5), etc. were made. Fig. 6 shows table of correlation-ship between refraction and ocular axial length noted in about 100 cases ranging from 18 to 40 years of age. This table reveals that the emmetropia is distributed over the area from 21.1 to 25.0 mm, average area of 23.1 mm.

Discussion

The ultrasonic generator has a momentary generating power of 5-6 w/cm² when manipulated by the pulse method, while it shows less than 2 places of decimals in w/cm²

Fig. 6. This is the correlation table between the refraction and the ocular axial length.



on the average when manipulated by the continuation method. Moreover, its contact with the cornea being restricted within 5 seconds causes no harm, as is indicated in comparison with Gilbert Baum's table[1].

We utilized the velocity in water for the measurement of that in parts of the eye, because the former is the most accurately established. For example, the velocity in water at 4°C shows 1.43×10^5 cm/sec, and that at 20°C indicates 1.4831×10^5 cm/sec. This time we utilized the velocity in water at 4°C.

In the measurement of ocular axial length Oksala A[3]. stated it to be 0.4 mm shorter than its actual length when it was thought that the eye was filled with water inside, disregarding the crystalline lens. In case of eyes examined by us the calculation based on the thought that they are filled with water inside proves to be about 3.5μ shorter per 1 mm than that from the velocity in the eye indicating 1.48×10^5 cm/sec. Accordingly, adoption of the average velocity in the eye is thought to assure of the accuracy in the measurement of ocular axial length. As to the appearance of echo in the lens, echo often appeared in the area supposed to be backward of lens, but there remains mnch to be accurately determined by further research.

Summary

The authors have succeeded in the measurement of ocular axial length by a pulsed ultrasonic echographic equipment and have found nothing that would cause an error in the measurement.

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ON THE REACTIVITY OF CHOROIDAL RETICULOENDOTHELIAL SYSTEM

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On the theory of the vital defense function of the reticuloendothelial system (RES) advanced by Aschoff (1913-24), Kiyono (1920) and others there have been many opinions voiced in the past. More recently, some noteworthy reports, particularly in the field of pathology, have been published on the variation of RE cells concerned with the origination of inflammatory cells.

In the realm of ophthalmology, too, since Goldmann (1909) reported on his study on vital staining of ocular tissues, not a few authors have followed up the results of his experiments under normal and various anomalous condition; in recent years, however, research in this line has become less active and even neglected from all appearances. At this juncture, it seemed to the present authors that a rescrutiny of the ocular RE cells as playing a fundamental part in occurrence of inflammation of the eye has become necessary in line with opinions concerning the vital defense function of RES. At present, endogenous uveitis presents the gravest problem of all inflammatory diseases of the eye. Its etiology is as yet shrounded in darkness. It is, therefore, very important to know what part the intrauveal RES play in the pathogenesis and type determination of uveitis. According to the results of our clinical study of uveitis patients, it seems incontestable that the majority of uveitis cases in recent years have been induced with general reactions as background. In consideration of these facts, the present authors were led to undertake an experimental study on the behavior of the ocular RES, with emphasis on comparison of this behavior with that of RES in other parts of the body. The results of this study showed that the choroidal RES exhibits a reactivity specific in some respects.

Materials and Methods

Mature white rabbits of about 2 kg body weight were used as experimental animals and 2.5% lithion carmin solution, 1% trypan-blue solution and 10% India-ink suspension (Pilot drawing ink) as staining pigments. The last item contained 60 mg of 0.1 μ carbon-black granules in 1 cc of suspension; this is applicable in vital staining.

For vital staining, 3.6 cc per kg of the above staining fluids was intravenously injected once daily, for 3 to 14 consecutive days as determined by the series of experiment. For activation of the RES in general, one of the following two methods, both reported by Akazaki as effective in inducing RE cell reaction, was applied: a) single intraperitoneal injection of 5 cc per kg of typhoid-paratyphoid vaccine and observation 10 days to 2 weeks thereafter; b) administration of the above-mentioned dosis of 1% trypan-blue solution for 10–14 days.

Clinically, slit-lamp microscopy and ophtalmoscopy were applied, and histologically, stretch preparations of the subcutaneous tissue of the back, the subconjunctival tissue and the choroid were made; stamp preparations of the liver, the spleen, the bone-marrow and the lymphatic gland were made; and celloidin-paraffin sections of the eyeballs and other organs fixed in Susa's solution were microscopically examined. These preparations were stained with Giemsa's stain, hematoxylin-eosin, hematoxylin or Kernechtrot.

Results

The distribution of RE cells in normal eyes was found nearly in accordance with the data given by Goldmann and many later authors. Under the optimum conditions for staining RE cells (as observed 24 hrs. after 5–7 intravenous injections of the above-defined

Table 1. The distribution of phagocytes in the normal eye when trypan-blue was administered intravenously.

Ectodermal	
Conjunctival epithelium	(−)
Corneal epithelium	(−)
Lens	(−)
Epithelium of iris and ciliary body	(−)
Retina	(−)
Optic nerve fibers	(−)
Mesodermal	
Tunica propria of conjunctiva and subconjunctival layer	(++)
Fascial sheath of eye-ball (Tenon's capsule)	(++)
Stroma and endothelium of cornea	(−)
Sclera	(+)
Iris	(±)
Pars plana of the ciliary body	(+)
Ciliary processes	(+++)
Choroid	(++)
Perivascular space of retina	(±)
Sheath and septum of optic nerve	(+)

dosis of lithion carmin or trypan-blue), the distribution in the normal eye, as shown in Tab. 1., was the densest in the ciliary processes, next in the choroid, the tunica propria of conjunctiva and the subconjunctival layer, the fascial sheath of the eye-ball, the pars plana of the ciliary body, the sheath and the septum of the optic nerve, the iris, and the sclera in descending order; distribution was poorest in the perivascular space of the retina. Of these parts, the uveal tract showed phagocytosis quite as intense as in the liver and the spleen.

Ink-granule phagocytosis in the choroid.

In the choroid vital-stained with trypan-blue or lithion carmin, the phagocytes were mainly found in the intervascular stroma, showing a beautiful tigroid appearance in stretch preparations. Besides, a small number of the cells in the endothelium of blood vessels were found taking in a little pigment, and sometimes were found fallen off into the vascular lumen. When India-ink was administered separately, however, unless the administration was repeated very frequently, the phagocytizing RE cells were limited to the reticuloendothelia such as the Kupffer's cells in the liver, the cells of the sinus endothelium of the bone-marrow and the reticulum cells in the pulp cord of the spleen, and no India-ink phagocytosis could be observed in any ocular tissue.

Now, after intravenous injection of a mixture of 2% trypan-blue solution and 20% India-ink-suspension in equal volumes, such a picture was found entirely replaced. Following daily injection of trypan-blue—the foremost representative of the diffusible pigment—mixed with India-ink—which emigrates less beyond the walls of blood vessels than any other pigment—India-ink granules began to appear on the 4th day in the fundus of the

eye along the blood vessels, growing denser with time and finally after a lapse of 10 days a pigment spot was found covering a wide area excluding the vascular portion of the fundus. Histological examination of preparations in this stage showed that cells had swollen with the saturation of India-ink; those that had taken in trypan-blue alone were found coexisting in all the layers of the choroid. Such an India-ink phagocytosis is a phenomenon specifically observed in the choroid of all ocular tissues but never in the iris, the ciliary body or the bulbar conjunctiva. This fact was combined by the complete absence of such a phagocytosis in the anterior part of the eye-ball as examined with a slit lamp. On the other hand, throughout the term of our observation, the histiocytes in the subcutaneous tissues never showed India-ink phagocytosis. Thus, in this series of experiments it was found, that the choroid alone showed a specificity in its behavior following administration of pigments separately or in mixture (Tab. 2).

Table 2. Phagocytosis of RE cells in some ocular and other organ tissues following administration of pigments separately or in mixture. The choroid alone shows a specificity in its behavior.

	trypan-blue	India-ink	trypan-blue with India-ink	
			trypan-blue	India-ink
Subconjunctival tissue	+	−	+	−
Iris and ciliary processes	+	−	+	−
Choroid	+	−	+	+
Subcutaneous tissue	+	−	+	−
Liver	+	+	+	+

Later on, the outer portion as well as the fundus gradually lost their blue tincture, so that they appeared quite normal in color, but the stain of India-ink phagocytosis remained in irregular shape formed by coalescense and expansion. Histological examination showed that, trypan-blue granules were absent in the choroid and cells with phagocytized India-ink granules were found remaining only in the lamina vasculosa and stroma, having faded away from the lamina choriocapillaris and the suprachoroids. The inked cells remaining were far fewer, than in the earlier stages and were found deprived of their original form and metamorphosed into so many lumps of ink grains.

Comparative study of ocular RES and general RES under activation.

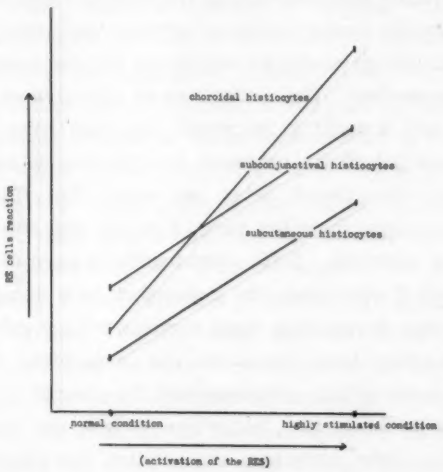
In the next stage, the experimental animals were subjected to the activation treatment described below, for investigating the behavior of ocular RES in the state of Akazaki-Kojima's so-called generally stimulated condition. In Group I, the animals were intraperitoneally injected with one dosis of 5 cc per kg of typhoid-paratyphoid vaccine, vital-stained with trypan-blue daily injected intravenously for 5 days starting 6 to 10 days after the vaccination and tissues from them were sampled 10 to 14 days thereafter. In

Group II, trypan-blue alone was intravenously injected daily for 14 consecutive days, and 24 hrs. after the last injection preparations were made of their tissues. As ordinary sections are not suitable for studying the morphology of RE cells, the samples were made into stretch preparations; of the ocular tissues, the choroid containing the pigmentary layer of the retina and the subconjunctival tissue were sampled (it was an onerous task indeed to stretch the anterior uvea!), and the subcutaneous tissue was taken to represent the RES in other parts. Rabbits injected only with trypan-blue 5 times, without previous activation, were used as control animals.

In comparison with the control animals, the experimental animals showed obvious activation in their subcutaneous tissue, which may be summarized as follows: 1) increase of RE cells, 2) proliferation of prehistiocytes, the precursors to histiocytes, 3) enlarged focal or cordal arrangement of histiocytes and 4) appearance of numerous free small round histiocytes.

The histiocytes beneath the bulbar conjunctiva are found more stimulated than the subcutaneous histiocytes in the same organism even in normality and are understood to fall under the notion of Rössle-Maximow's physiological inflammation, but with the advance of general activation, the former began to show even higher activity, the rate of rise in activity running nearly parallel with that of the subcutaneous histiocytes (Tab. 3).

Table 3. Cellular reaction of the RES under generally stimulated condition.



The choroid being so tenuous as to make stretching very difficult, in the stained preparations, we often found individual cells in it overlapping one another, so that the cytological examination is placed under far less favorable conditions, than in examination of the subcutaneous and the subconjunctival tissues. So, we failed here to obtain the findings specific to the stimulated state, such as observed in the subcutaneous tissue

above: on the other hand, the conditions were rather favorable for estimating the changes in the cell count per unit volume.

Under normal conditions, the number or density of phagocytes in the choroid is not necessarily large, but in activity, they may be placed between the subconjunctival and the subcutaneous histiocytes. However, once the activation runs high, pigment phagocytosis strong enough to cause appearance of blue dots around the choroidal blood vessels becomes noticeable by ophthalmoscopy, and the stretch preparations show the picture of countless phagocytes taking up all the space outside the vascular lumina. Such an abrupt increase-rate of choroidal histiocytes is never exceeded by that of subcutaneous or subconjunctival histiocytes under intense excitation (Tab. 3); besides, the arrangement of the proliferated phagocytes densely aggregated in the intervascular spaces more or less vividly reminds us of the abovementioned focal or cordal arrangement and contains not a few small round cells. Thus, it was ascertained, that the choroidal RE cells are, under activation, proliferated far more rapidly than the subcutaneous histiocytes and the RE cells in other regions and can even show the characteristics of the RE cells under stimulation. Such findings are hard to arrive at with ordinary preparations alone; they are only given by stretched preparations of the choroid as used in this study.

Discussion

As factors causing uveitis and determining the clinical types of the disease, especially those providing the respective characteristics of anterior and posterior uveitis, mention is made of local anatomical and physiological conditions, but the ocular RES also comes into the question in this connection. The ocular tissues are quite as rich in RE cells as the tissues of other organs, and it ought to be noted, that the uvea is particularly rich in them. That in the uvea the ciliary processes and the choroid are particularly abundant in RE cells may be easily understood, when we recall that these parts form barriers which act powerfully in maintaining the visual function and represent sites where metabolism is carried on very intensely. Some recent authors have opined that vital staining is, so to speak, a method of evaluating the metabolism in a living organism by means of pigment-labeled protein, say, trypan-blue must combine with α-gobulin, albumin and such serum protein fractions before being taken into the phagocytes. Now, when we compare the density of RE cells in the ciliary processes and the choroid, it is indeed considerably lower in the latter than in the former, but when we take the volume of the choroid and its very intense reactivity under activation into account, the phagocytotic capacity of the choroid as a whole must be far larger than that of the ciliary processes—as large as to permit it taking the leading role in the intraocular phagocytosis.

The phagocytosis of India-ink granules administered in mixture with trypan-blue by the choroidal phagocytes is a very peculiar behavior specific to all the RE cells in all other parts of the body, but we are aware of no report containing any statement or discussion on this phenomenon. We are not yet in the clear as to why such a

phagocytosis should be found in the choroid, but seeing that, whereas no ink granule was found emigrating from the blood vessels as long as the ink was administered separately, when it was adminstered in a mixture with trypan-blue, the phagocytes in the stroma could take up ink granules, we may in the first place infer that perhaps the action of trypan-blue, in injuring the vascular walls, facilitates the emigration of the India-ink granules. But when we come to the question of why the chorcid alone of all tissues offers the scene of such a phenomenon, the matter becomes further complicated. The peculiarity may lie in the specific state of the vascular system or the blood flow there or in the specificity of the choroidal RE cells themselves, but we are not in position to tell which at present. Nevertheless, this specific behavior shown by the choroid as a whole must be noted with due emphasis, for we might suppose that, though our experiments were concerned with the phagocytosis of foreign matter in the blood flow, the choroid may show a specific reactivity also against the various phlogogenic substances coming in hematogenously, including bacteria, virus and antigens, and this specificity in behavior may give a characteristic form to the pathology or type of choroiditis.

Next, in the case when a RE cell comes upon foreign matter beyond its power of disposition, sometimes it may sensitively react, either by proliferating or by amplifying the metabolic mechanism in its body, for the cause of defending the organism from injury. It is said that the state described above of highest cell proliferation and metamorphosis representing RES reactions induced by intense vital staining and general activation is similar to the state observed in inflammatory disease. Akazaki-Kojima and their school have summarized the histological findings of the subcutaneous tissues under generally stimulated condition into 1) increase of RE cells in number, 2) proliferation of prehistiocytes, 3) enlargement of focal or cordal arrangement of histiocytes and 4) appearance of numerous free small round histiocytes; and having succeeded in transforming proliferated RE cells into free round cells of the forms of lymphocytes, plasmacytes, monocytes and giant cells, they have pointed out the necessity of searching for the origin of mononuclear cells in inflammatory foci from the angle of RE cells as well.

As already stated above, the experiments presented herein have served in demonstrating the capacity of the choroidal RES too, under general activation of RES, in effecting increase of its cell number and forming focal or cordal arrangements and small round cells from its cells. Here, however, we face the question whether the source of this immense increase of the RE cells can be ascribed to blood, instead of tissues. But this possibility seems to be negligible, if we recall, that during our experiments, scarcely any cell in the peripheral blood and in the choroidal vessels was found to show notable pigment phagocytosis. It may be cited in its connection also, that Akazaki and Kojima, in their study on the subcutaneous histiocytes found, that the above-mentioned process of proliferation and metamorphosis of histiocytes could be carried on without the help of blood cells, and that this process can be reproduced in vitro too.

In short, we can say, that the sensitive reaction of the choroidal RES to general

Fig. 1. Stretch preparation of subcutaneous tissue of rabbit after 5 days' administration of trypan-blue. (Unstained.) Histiocytes barely observable.

Fig. 2. Stretch preparation of subcutaneous tissue of rabbit after 14 days' administration of trypan-blue. (Unstained.) Histiocytes clearly outlined by trypan-blue granules. Nuclei in the round lighter area. Cells focally proliferated; some cells turned small and round.

Fig. 3. Stretch preparation of bulbar subconjunctival tissue after 5 days' administration of trypan-blue. (Unstained.)

Fig. 4. Stretch preparation of bulbar subconjunctival tissue after 14 days' administration of trypan-blue. (Unstained.) Very notable proliferation of histiocytes, suggestive of focal or codal formation.

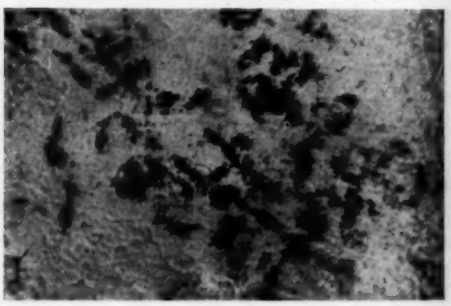


Fig. 5. Stretch preparation of choroid after 5 days' administration of trypan-blue. (Unstained.) Relatively few histiocytes.

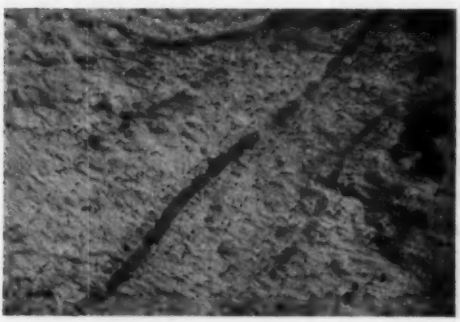


Fig. 6. Ditto under higher magnification.

Fig. 7. Stretch preparation of choroid after 14 days, administration of trypan-blue. (Unstained.) Very notable proliferation of histiocytes, suggestive of focal or cordal formation.

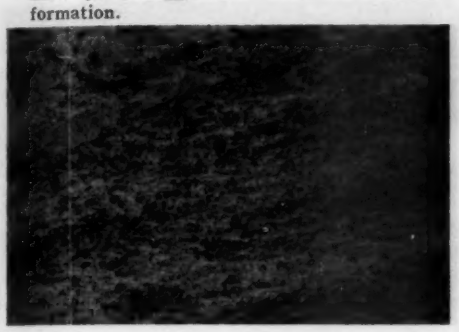


Fig. 8. Ditto under higher magnification.

Fig. 9. Stretch preparation of choroid after 5 days' administration of India-ink plus trypan-blue. (Unstained.) Black dots: Histiocytes that have taken up India-ink granules. Grey dots: Histiocytes that have taken up trypan-blue.

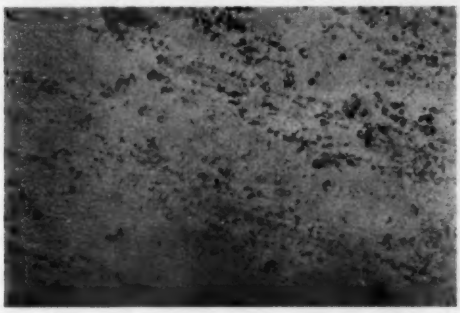


Fig. 10. Stretch preparation of choroid after 10 days' administration of Inkia-ink plus trypan-blue. (Unstained.) Cells with phagocytized India-ink granules much more numerous than in the above.

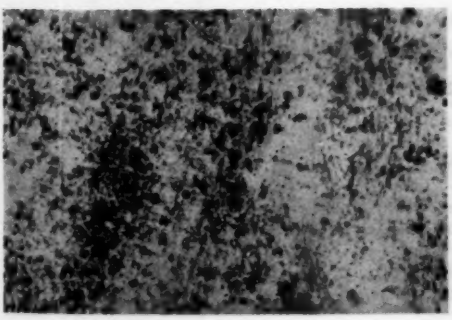


Fig. 11. Stretch preparation of choroid 6 months after 14 days' administration of India-ink plus trypan-blue. (Giemsa's staining.) Grey dots: Cell nuclei have affinity to Giemsa's stain. Trypan-blue already faded.

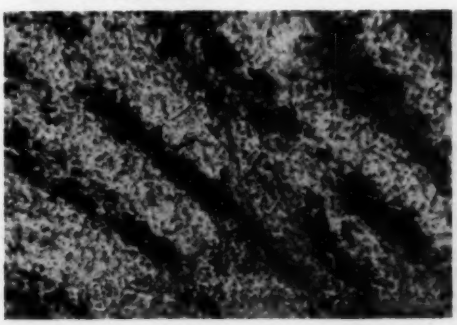


Fig. 12. The ocular fundus photographed after 13 days' administration of trypan-blue.

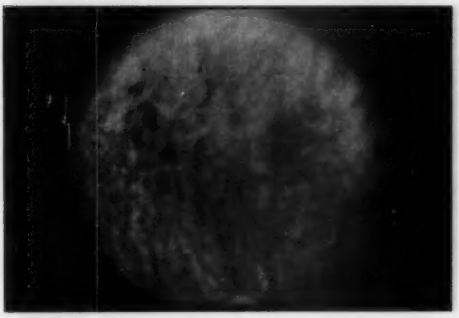
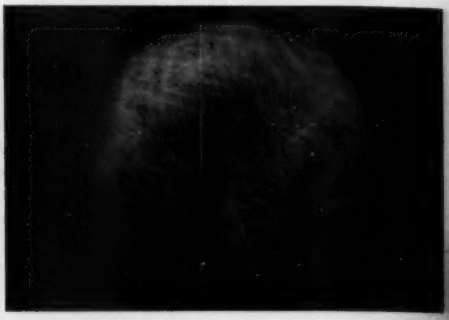


Fig. 13. The ocular fundus photographed 3 months after 14 days' administration of trypan-blue plus India-ink.



stimulation is an exceedingly interesting datum in discussing the nature of uveitis as a systemic disease. This is especially so, when we view such a choroidal reaction in correlation with the function of RES in producing antibodies. Such a highly sensitive reactivity or excitability exhibited by the choroid may afford a valuable keystone for founding a theory to elucidate the problems concerning the etiology and pathogenesis of uveitis.

Conclusion

1. Cells belonging to the reticuloendothelial system (RES) are found in the uveal tissues including the ciliary processes and the choroidal membrane as well as in the bulbar conjunctiva, quite as abundantly as in any other region of the body.

2. The choroidal RE cells notably phagocytize India-ink granules administered in mixture with trypan-blue. This shows a specific behavior of these cells dissimilar to the phagocytic action of RE cells in any other region, and may perhaps represent a factor determining the nature of the choroiditis.

3. Under general activation of RES, the choroidal RES reacts more sensitively than RES in any other part, and in stretched preparations the choroidal RE cells are seen immensely proliferated, manifestly showing the characteristics of such cells under stimulation. This finding suggests a close correlation existing between the excitability of the choroid and the pathogenesis of uveitis as a systemic disease.

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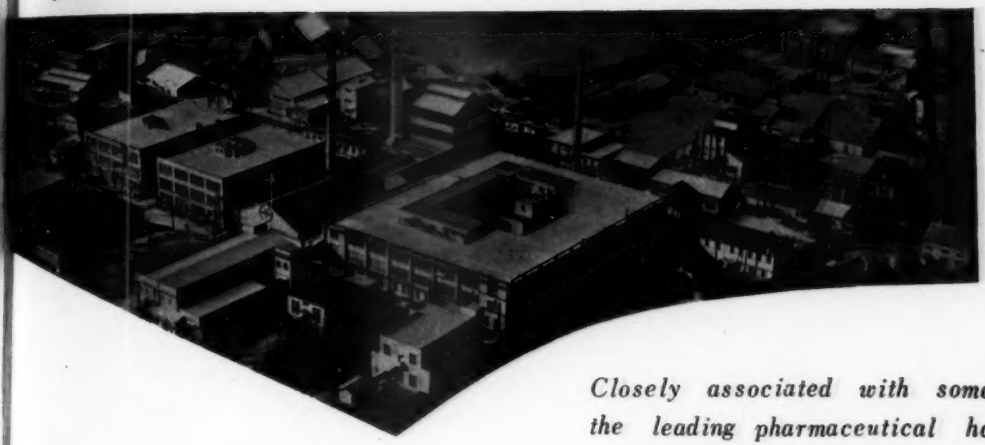

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